SHOCK INDUCED JADOMYCIN PRODUCTION FROM *STREPTOMYCES VENEZUELAE* ISP5230 VS1099

by

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ABSTRACT

The production of jadomycin by *Streptomyces venezuelae* was carried out by applying various environmental stress conditions on the culture in nutrient deprived, amino acid rich MSM production media. The effects of culture transfer time (18, 21 and 24 h) from the growth media to the production media, heat shock at different temperatures (35, 40, 45 and 50°C) and exposure times (0.5 and 1 h), alcohol shock using ethanol and methanol at various concentrations (3.0, 4.5, 6.0 and 7.5 % v/v), multiple shocking by 3 % v/v ethanol at various times [once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h)] and ethanol shock with various concentrations (3.0, 4.5 and 6.0 % v/v) and varying nitrogen concentrations (45, 60 and 75 mM) in the media on the growth and activity of *S.venezuelae* and jadomycin production were investigated. The growth of *S.venezuelae* was determined by measuring cell number and TF yield and the Jadomycin B was determined by measuring the optical density (AU¬526nm). Shocking the culture that was transferred after 18 h of growth in the growth medium produced the highest jadomycin, cell growth and activity compared to those that were transfered after 21 and 24 h. The culture shocked at a temperature of 40°C for 1 h produced the highest jadomycin. Higher temperature and/or longer exposure time resulted in poor cell growth and activity. The ethanol concentration of 4.5 % v/v produced the highest jadomycin. The culture shocked with ethanol once (0 h) produced higher jadomycin, cell growth and activity compared to the cultures shocked twice [(0, 3 h) and three times (0, 3 and 6 h)]. A nitrogen concentration of 75 mM with 4.5 % v/v in the production media increased cell growth and jadomycin production. The results showed that shocking the culture once with ethanol (3 % v/v) produced 81.5 % more jadomycin than shocking with heat at a temperature of 45°C for 1 h. However, increasing the ethanol concentration to 4.5 % v/v produced 10.8 % more jadomycin than 3 % v/v ethanol. By increasing the nitrogen concentration to 75 mM in the production medium and shocking with the 4.5 % v/v ethanol further increased the jadomycin production by 13.9 % above that without nitrogen addition. Therefore, the treatment with ethanol (4.5 % v/v) and nitrogen (75 mM) is recommended for jadomycin production.

LIST OF ABBREVIATIONS AND SYMBOLS USED

A.U.	Absorbance Units
Ala	Alanine
ATP	Adenosine Triphosphate
B	Isoleucine
C	Carbon
CFU	Colony Forming Units
DF	Degrees of Freedom
G	Glycine
H	Hydrogen
H_3BO_3	Boric Acid
HCl	Hydrochloric Acid
HEMPG2	Cancer Cell Line HEMPG2
IC_{50}	50% Inhibitory Concentration
IM-9	Cancer Cell Line IM-9
jadR₁	Gene *jadR₁*
jadR₂	Gene *jadR₂*
MDA-MB-435	Breast Cancer Cell Line MDA-MB-435
MOPS	3-(N-morpholino)propanesulfonic Acid
MS	Mean Sum of Squares
MSM	Mineral Salt Medium
MYM	Maltose-Yeast Extract-Malt Extract
NaCl	Sodium Chloride
O	Oxygen
R	Ribosomes
SS	Sum of Squares
SV1	*Streptomyces venezuelae* Infecting Phage
T	Threonine
T-47D	Breast Cancer Cell Line T-47d
TF	2, 3, 5-Triphenyl Formazan
Tris	Tris(hydroxymethyl)aminomethane Buffer
TTC	2, 3, 5-Triphenyl Tetrazolium Chloride
V	Valine

$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	Ammonium Molybdate
$C_{25}H_{23}NO_{10}$	Jadomycin S
$C_{26}H_{23}NO_9$	Jadomycin G
$C_{27}H_{25}NO_9$	Jadomycin Ala
$C_{28}H_{27}NO_{10}$	Jadomycin T
$C_{29}H_{29}NO_9$	Jadomycin V
$C_{30}H_{31}NO_9$	Jadomycin B
$C_{33}H_{29}NO_9$	Jadomycin F
$C_6H_{12}O_6$	Glucose
$C_6H_{13}NO_2$	Isoleucine
$C_7H_{15}NO_4$	MOPS Buffer
$CaCl_2$	Calcium Chloride
$CuSO_4 \cdot 5H_2O$	Cupric Sulfate
$FeSO_4 \cdot 7H_2O$	Ferrous Sulfate
H_3BO_3	Boric Acid
HCl	Hydrochloric Acid
$MgSO_4$	Magnesium Sulfate
$MnSO_4 \cdot 4H_2O$	Manganese Sulfate
$NaCl$	Sodium Chloride
$NaH_2PO_4 \cdot H_2O$	Sodium Phosphate Monobasic
$NaOH$	Sodium Hydroxide
$ZnSO_4 \cdot 7H_2O$	Zinc Sulfate

ACKNOWLEDGEMENTS

CHAPTER 1. INTRODUCTION

Many drugs used around the world have their beginning in natural products. For example, penicillin and other drug metabolites which are commonly used to cure infection or inflammation have been obtained from naturally occurring organisms (Drews, 2000). At the microbial level, many microbes have gradually upgraded their entire biochemical pathways to compete with species around them. These pathways are called secondary metabolic pathways and the products obtained are referred to as secondary metabolites. These have been exploited widely by researchers over many generations for their toxic characteristics and antimicrobial properties (Dupuis, 2010; Hayat et al., 2007).

Streptomyces are soil bacteria abundant in the environment and capable of producing 60% of the known antibiotics. Some commonly produced clinical antibiotics from *Streptomyces* species are shown in Figure 1.1. *Streptomyces* are fungus like filamentous bacteria known to produce at least one secondary metabolite during their growth when exposed to environmental stress condition (heat shock, ethanol treatment or phage infection) or subjected to nutrient limited conditions in their media (Izard, 2001; Watve et al., 2001; Jakeman et al., 2006; Burdock et al., 2008).

Streptomyces venezuelae is known to produce two antibiotics. Chloramphenicol is produced when *S.venezuelae* undergo unbalanced growth in a nutrient-deprived environment (Wang and Vining, 2003). The jadomycins are secondary metabolites that are produced when *S.venezuelae* are grown in a nutrient-deprived, amino-acid rich environment and are stressed with an environmental shock, such as heat treatment, ethanol treatment or phage infection (Ayer et al., 1991; Wang and Vining, 2003). Jadomycins exhibit activities against bacteria, yeasts and fungi, as well as exhibit cytotoxic properties toward cancer cells. These properties make jadomycin production very important.

Production of jadomycin essentially needs three phases of *S. venezuelae* growth and sporulation. The primary phase is the growth of *S. venezuelae in* a nutrient rich environment (maltose-yeast extract-malt extract (MYM) broth). This is done to obtain a large number of healthy bacteria. The second phase involves the introduction of *S.venezuelae* to nutrient
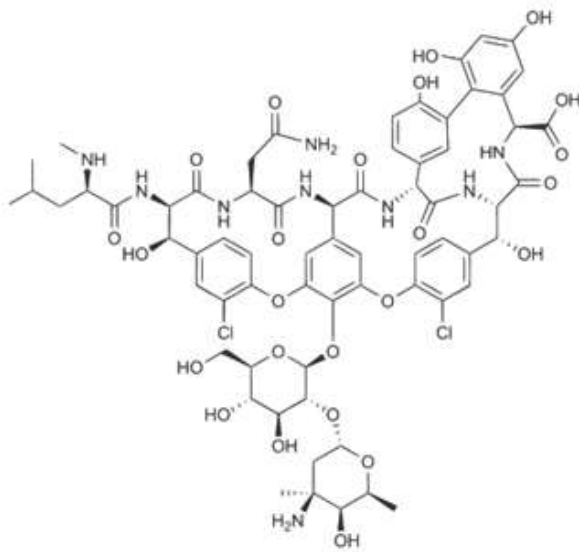
(a) Vancomycin from *S. orientalis*
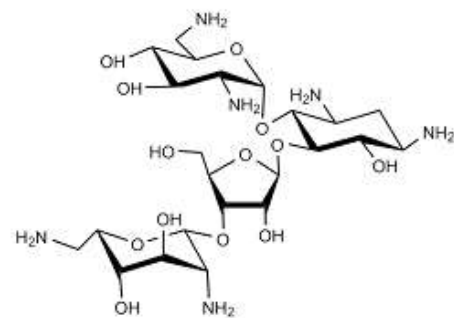
(b) Neomycin from *S. fradiae*

(c) Chloramphenicol from *S. venezuelae*
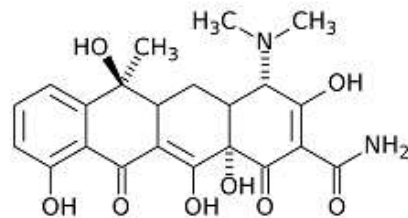
(d) Tetracycline from *S. rimosus*

Figure 1.1. Commonly produced clinical antibiotics from *Streptomyces* bacteria (Dupius, 2010).

deprived-amino acid rich production media and shocking the bacteria immediately in the production media, thereby subjecting the organism to stress in order to induce transition from vegetative cells to spores. The final phase is production of jadomycin from the production media during the course of transition. The time of applying the stress condition is very important in the process of jadomycin production in order to achieve high yield. In addition, shocking must be done to high numbers of young cells, which ultimately leads to good production of the jadomycin.

The dehydrogenase measurement test is a potential tool in determining the quantity of live bacterial cells and optimal time for shocking to start jadomycin production. The dehydrogenase enzyme (present within the cells) oxidizes the organic content within bacteria and can be measured using tetrazolium salts. In the presence of these enzymes, triphenyl tetrazolium chloride (TTC) is reduced to triphenyl formazan (TF) to produce a red coloured solution (Mahmoud and Ghaly, 2006; Burdock et al., 2010). This method can be employed during the bacterial growth to determine the optimal time for inoculation and for initiating the shocking process to start the jadomycin production.

CHAPTER 2. OBJECTIVES

The main focus of this study was to evaluate the effectiveness of nutrient deprived and environmental stress conditions on the production of the secondary metabolite, jadomycin B from *Streptomyces venezuelae* ISP5230 VS1099. The specific objectives were to:

1. Study the growth of *Streptomyces venezuelae* in MYM broth media.

2. Determine the optimum time for *S.venezuelae* culture transfer from nutrient rich MYM medium to amino acid rich MSM production media.

3. Determine the optimal conditions for shocking *Streptomyces venezuelae* to induce jadomycin production.

    (a) Study the effect of heat shocking by various temperatures (35°C, 40°C, 45°C and 50°C) on *Streptomyces venezuelae* for initiating jadomycin B production.

    (b) Study the optimal shock time (0 and 3h) and effectiveness of alcohol shock using two different types (ethanol and methanol) at different concentrations (3%, 4.5%, 6 and 7.5%) on *Streptomyces venezuelae* for initiating jadomycin B production

    (c) Study the effect of multiple shocking using 3% absolute ethanol at various times after the inoculation of MSM media (0 and 3 h) hour and at (0, 3 and 6 h) on *Streptomyces venezuelae* for initiating jadomycin B production.

    (d) Study the effect of nitrogen concentration (45 mM, 60 mM and 75 mM) in MSM production media while shocking with various concentration of ethanol (3%, 4.5%, and 6%) on *Streptomyces venezuelae* for initiating jadomycin B production.

4. Perform a comparative analysis on the various shocking processes to determine the most effective one for jadomycin production.

5. Determine *Streptomyces venezuelae* dehydrogenase activity during growth in nutrient rich media, acclimatization to the amino acid rich production media, after shocking in the production media and during jadomycin B production in the production media under optimum growth and shocking conditions.

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CHAPTER 3. LITERATURE REVIEW

3.1. *Jadomycin*

Jadomycins are neutral hydrophobic molecules belonging to a unique family of benzoxazolophenanthridine antibiotics produced by the *Streptomyces* species (Jakeman et al., 2009; Rix et al., 2004; Burdock et al., 2011). Jadomycins are complex molecules containing five fused rings, (A through E), one of which possesses an oxazolone ring of amino acid side chain (Figure 3.1). They are angucycline four ring structures with a sugar moiety connected to the fourth ring and having amino acid integrated into the structure in order to form an oxazolone ring (Burdock et al., 2011; Kirschning et al., 2000). Jadomycins are brilliantly coloured dark reddish compounds that exhibit promising anticancer and antibiotic activities (Ayer et al., 1991; Doull et al., 1993). The intense colour formation is due to the integrated multiple aromatic rings present in them. Jadomycin B has an integrated amino acid, isoleucine in its structure. A product description of jadomycin B is given in Table 3.1.

The jadomycins have different types of lipophilic and hydrophilic portions and larger molecular weights greater than five hundred. Analyses using different type of bacterial strains and cancer cell lines revealed the ability of jadomycins to act potentially against gram positive bacteria and to possess anticancer activity (Ayer et al., 1991; Zheng et al., 2005). This secondary metabolite also contains sugar functionality, namely a L-digitoxose group as shown in Figure 3.1. These angucycline classes of antibiotic have a common biosynthetic origin as evident from their backbone structure consisting of the four-ring frame with aglycone moiety (Zhou et al., 2010; Dupuis et al., 2011).

3.2. *Jadomycin Biosynthesis*

Jadomycins are synthesized in the presence of enzymes that tend to make up the pathway for secondary metabolites in the organism. The enzymes participating in this biosynthesis are coded for genes that are measured in clusters along the bacterial chromosome (Rix et al., 2003; Doull et al., 1994). When *Streptomyces venezuelae* ISP5230 are exposed to environmental stress like heat or ethanol, the entire pathway is stimulated.
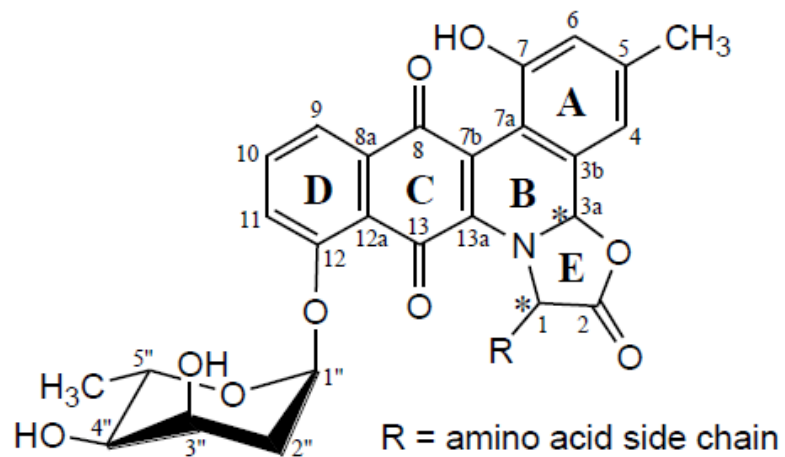
Figure 3.1. Jadomycin general structure indicating four aromatic rings marked as A, B, C and D with variable R-group and L-digitoxose group. The atoms are numbered according to the scheme used for all jadomycins. The stereo-centers of the oxazolone ring are marked with asterisks (*) (Dupuis, 2010).

Table 3.1. Jadomycin product description (ACC Corporation, 2011).

Name	Jadomycin B
PKS Type	Type II
Classification	Angucycline
Starter Unit	acetyl-CoA
Chain Length	10
Sugar Unit	L-digitoxose
Activity	Antibacterial
Composition	$C_{30}H_{31}NO_9$

Numerous studies were carried out to identify the genes and enzymes that are actively involved in jadomycin biosynthesis pathways (Dupius, 2010; Han et al., 1994; Yang et al., 1996; Wang et al., 2001; Wang et al., 2002; Chen et al., 2005). The corresponding proteins of these active genes (JadA, JadB, JadC etc.,) were identified and categorized as shown in Figure 3.2. These genes and proteins have differential functions in catalyzing the biosynthesis pathway (Wang et al., 2002; Cottreau et al., 2009).

The jadomycin biosynthesis pathway proposed by Jakeman et al (2005) is shown in Figure 3.3. The mechanism starts with synthesis of the polyketide core, nine malonate and single acetate molecules. The polyketide cluster made up of proteins (JadA, JadB and JadC) combine these substrates into polyketide chain (11). Secondly, the JadD, JadE and JadI reclaim this chain into rings, thereby resulting in the production of compound (12). Then, the proteins JadF, JadG and JadH result in the hydrolysis and aromatization process to generate a reactive aldehyde intermediate (13). This intermediate reacts with the amino acid to produce an imine (14) and this undergoes decarboxylation process and cause the ring closure to produce an aglycone form of the secondary metabolite (15). Finally, by the glycosyltransferase (JadS), jadomycin is glycosylated at position C12.

This overall pathway reveals the number of genes that tend to code the enzymes involved in jadomycin biosynthesis. However, it has failed to exhibit any candidate genes for enzymes which tend to catalyze the incorporation of amino acids. It was proposed that this step is chemical, not enzymatic, and the incorporation of amino acids occur spontaneously (Chen et al., 2005; Wang et al., 2003; Yang et al., 2001).

3.3. *Secondary Metabolite Production*

The first known secondary metabolite isolated and characterized from the jadomycins were: jadomycin A and jadomycin B (aglycone form) (Ayer et al., 1991; Doull et al., 1993; Jakeman et al., 2006; Burdock et al., 2008). Jadomycin B is based on the amino acid isoleucine, while other types of jadomycin can be derived using various types of amino acids as shown in Table 3.2, where the position of the R-group is shown in Figure 3.1.
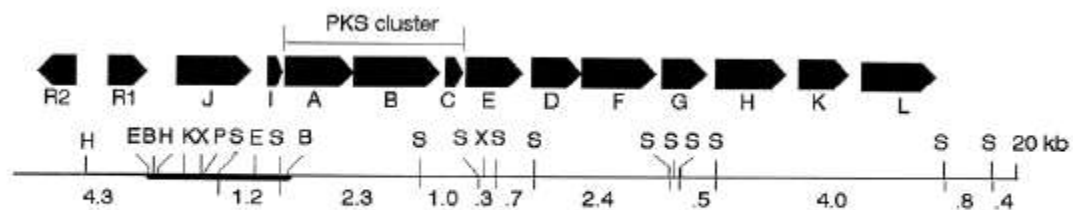
(a) Polyaromatic backbone with genes activity in biosynthesis
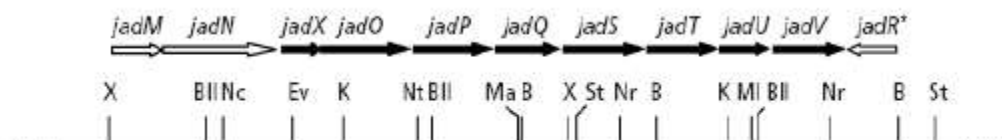
(b) Genes activity in dideoxysugar segment

Figure 3.2. *Streptomyces venezuelae* ISP5230 chromosome segments exhibiting the genes participating in biosynthesis pathway (Han et al., 2000; Wang et al., 2002).
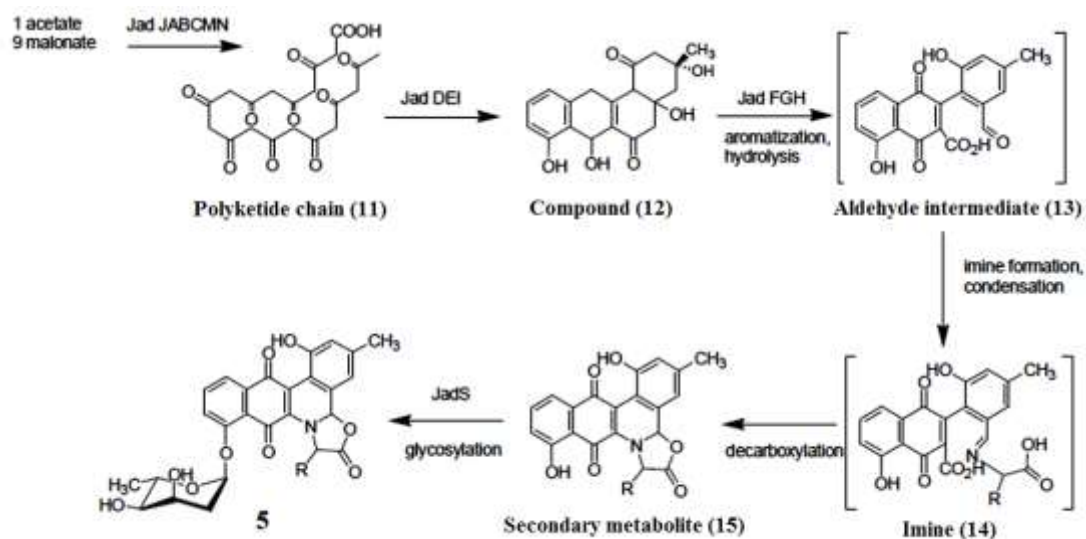
Figure 3.3. Proposed jadomycin biosynthesis pathway (indicated in arrows) and the enzymes involved in each steps (Jakeman et al., 2005).

Table 3.2. Jadomycin type and structure of R group based on amino acid used (Jakeman et al., 2008).

Jadomycin Type	Structure	Amino acid
Jadomycin B	R = H3C CH3	L-Isoleucine
Jadomycin DT	HO CH3	D-threonine
Jadomycin S	HO	L-serine
Jadomycin F		L-phenylalanine
Jadomycin G	H	Glycine
Jadomycin L	H3C H3C	L-leucine
Jadomycin DS	HO	S-serine
Jadomycin DM	H3C-S	D-methionine
Jadomycin T	HO CH3	L-threonine
Jadomycin Y	HO	L-tyrosine

The production of jadomycin takes place under specific conditions (heat induced stress, alcohol induced stress, nutrient depriving conditions or phage infection) all of which can result in the production of a secondary metabolite in the form of a coloured glycosylated compound. Some stress proteins are frequently induced in response to either a nutrient deprived condition or the cell damage caused by these physical parameters, showing the regular degree of overlap in the genetic system corresponding to the several configurations of stress (Lindquist, 1986).

The genome belonging to a mutant strain of *Streptomyces venezuelae* was shown to contain two oppositely adjusted open reading frames that act during the biosynthesis of jadomycin B. The gene, jadR2 had the same properties as repressor genes, which act during the unstressed condition to prevent the jadomycin production, whereas the other gene jadR1 acts as a positive promoter for jadomycin production. High expression of the gene jadR1 may interfere with the growth of the bacteria and cause potential inhibition. These two genes collectively accomplish a system of interacting stress-responsive regulatory network (Yang et al., 2001).

During chloramphenicol production conditions, a very little jadomycin is produced and no chloramphenicol is produced during jadomycin production conditions (Doull et al., 1994; Rix et al., 2004). It is speculated that a seperate type of regulatory system is engaged to give each secondary metabolite by the *Streptomyces venezuelae* (Doull et al., 1994; Rixet al., 2004). The levels of these natural products can be measured by spectrometric absorbance at specific λmax values. With reliable production and analytical methods in place, observations on different effective stress factors have been analyzed (Dupius, 2010).

3.3.1. *Nutrient Deprived Stress*

The deprivation of an important nutrient source such as carbon, phosphate or nitrogen source within the growth culture medium of different types of *Streptomyces* allows for the expression of genetic information to promote antibiotic biosynthesis (Doull and Vining, 1992). Doull et al. (1990) stated that the antibiotic production rarely appears during the rapid

growth in the nutrient rich media and the biosynthesis onset of antibiotics generally coincides with the decrease in the growth rate following the depletion of carbon, phosphate or nitrogen. Linking the genetic control mechanism which triggers antibiotic production with the universal regulatory networks which detect the fluctuations in available nutrients, allows for manipulation of metabolic actions (Liras et al., 1990).

The expression of the genetic information for antibiotic biosynthesis has been related to the control mechanism (the Pho system), which is responsible for identifying and adjusting cells to the readily obtainable nutrients in the environment (Wezel et al., 2011). Pho P is the response regulator which binds to DNA and controls the genes active in transcription belonging to the so-called pho regulon. This response regulator was shown to influence the expression of both primary and secondary metabolite genes in *Streptomyces* by exhibiting their influence over the nitrogen metabolism in *Streptomyces venezuelae* (Garcia et al., 2009).

The work by Jakeman et al. (2005 and 2006) involved alternate ways of stress induction by means of manipulating carbon, phosphate and other sources in the production media and growth medium to aid in stimulating secondary metabolite production efficiently. They investigated improved methods for jadomycin B production involving ethanol stress and alternate nutrient sources and suggested an alcohol shock of 3 % v/v concentration with glucose as a source of carbon (instead of galactose).

Jakeman et al. (2006) and Doull et al. (1994) analysed the effect of nitrogen deprivation (L-isoleucine) on jadomycin production in galactose-isoleucine production media in order to minimize the waste of amino acids. The result showed that an increase in amino acid from 15 mM to 75 mM resulted in greater yield of secondary metabolite production thereby indicating that the amino acid is incorporated directly into the jadomycin precursor molecule during biosynthesis pathway. An increase in the jadomycin production was seen with higher concentrations of L-isoleucine and a nitrogen concentration greater than 45 mM did not change substantially.

Glazebrook et al. (1990) reported that increasing the nitrogen concentration decreased the

rate of sporulation which resulted in a gradual increase in biomass yield. They also observed that sporulation was suppressed in the presence of increased nitrogen concentration (casamino acids and asparagine) and concluded that spore formation is greatly influenced only in nitrogen limiting conditions. Shapiro and Vining (1983) stated that limiting the nitrogen concentration to 30mM and having an excess of the carbon source during the growth period of *Streptomyces venezuelae* favoured sporulation.

3.3.2. *High Temperature Stress*

Heat shock proteins are a group of proteins whose synthesis is induced or accelerated upon exposure to higher temperature. While the significance and cellular function of the heat shock proteins is not clearly known, some general evidence describing their role in thermotolerance, protein degradation, synthesis of the macromolecules as well as in cell divisions have been reported (Neidhardt and Van Bogelen, 1987). Heat stress can cause modification in the enzymatic activity, reduction in the salt tolerance and degradation of RNA and ribosomes (Qoronfleh et al., 1989).

Doull et al. (1993) reported that when *Streptomyces venezuelae* were grown at temperatures between 37 and 42°C for an hour on a defined medium, a reddish glycosylated compound called jadomycin B was produced. The production of this antibiotic at higher temperatures and its absence from cultures grown at the 27°C showed that its synthesis may be linked to the heat-shock response, a general stress condition or the response marked by the production of the heat shock proteins. Secondary metabolite production can also be brought about by slowing the growth rate when exposed to the heat stress (Young et al., 2005; Wang and Vining, 2003). This deprivation can cause the organism to synthesize a certain set of proteins and causetheir transition from the vegetative cells to spores (Arnosti et al., 1986; lee et al., 1983; Yang et al., 1995). Ayer et al. (1991) found that *Streptomyces venezuelae* ISP5230 can produce chloramphenicol and also has the the capacity to synthesize jadomycin at elevated temperatures.

Doull et al. (1993) investigated the heat shock effect at different times post inoculation by measuring the jadomycin B titers 12 hours after the treatment. The results showed the

production to be rapid for the first 12 hours following the heat shock treatment and very little jadomycin was produced without this environmental stress condition. They also observed a lack of success in jadomycin production during the growth of the *Streptomyces venezuelae* ISP5230 under the production condition for chloramphenicol. The two antibiotics have different principle controls as there was no detection of the chloramphenicol in jadomycin producing cultures exposed to heat-shock.

Doull et al. (1994) observed that some accumulation of jadomycin B occurred at 37-42°C, but higher jadomycin production was seen when the cultures were grown at 27°C followed by heat shock at 42°C for 1 hour. The production was rapid for the first 12 hours after which the rate slowed and the concentration decreased throughout the following 96 hours. The timing of the heat treatment followed by the inoculation into the production medium has great influence over the jadomycin titers.

Guglielmi et al. (1991) suggested that the heat shock induced chaperones might play a significant role in the assembly of the multi-enzyme compound required for the antibiotic biosynthesis in the variety of the *Streptomyces*. GroEL chaperonins (heat shock proteins) play an important role in metabolite export and multi-enzyme complex assembly involved in the polyketide antibiotic production. The other highly preserved proteins such as DnaK and other conserved proteins have greater relation with the heat stress may be involved in the morphological development of *Streptomyces* (Bucca et al., 1991).

3.3.3. *Ethanol Stress*

Qoronfleh et al. (1990) stated that ethanol treatment resembles the effect of heat in terms of triggering heat shock proteins induction. Ethanol treatment is considered to be a key factor in actively promoting the release of secondary metabolites or increasing the membrane permeability. Ethanol stress is a key source of polyketide precursors, which inhibits the growth of *Streptomyces venezuelae*.

Doull et al. (1993) observed that when the culture was shocked with the ethanol, jadomycin B production was longer in time when compared to that with the heat shock. The maximum amount of jadomycin was obtained at the beginning and started to decline after 48

h. Doull et al. (1994) also reported that the ethanol treatment caused reduction of (or completely inhibited) the growth of the *Streptomyces venezuelae* and caused a greater reduction in the chloramphenicol production.

The growth of *S.clavuligerus* NPI strain in the presence of the alcohols was analyzed by Demain et al. (2001) in order to determine the effect of alcohol on the resting cells, with regard to their ability to transform the penicillin G into Cephalosporin type antibiotics. When the culture was grown in MT (MST less starch) or Mineral starch media (MST), they formed typical mycelial masses of interlaced hyphae. However, when the medium was supplemented with the ethanol, a difference in the morphology was observed. In the presence of 1% ethanol, the hyphae appeared more dispersed than the control medium, whereas in the presence of 2% ethanol, the hyphae appeared more extensively fragmented and dispersed. The growth under the 1 % ethanol or less than 2% methanol condition was less than in the MT medium, whereas with the 2 % ethanol shock the growth was severely restricted. Concentration higher than 2% of ethanol totally inhibited the growth. However alcohol addition at later stages during growth (2, 6 and 12 hours) did not have any influence on the bioconversion.

Higher level of jadomycin titers were reported to be accumulated in cultures in response to the ethanol, which is a common inducer of heat shock response in many different cell systems (Arnosti et al., 1986; Ayer et al., 1991; Lee et al., 1983; Qoronfleh., 1990).

3.3.4. *pH Stress*

Kontro et al. (2005) found that the pH for growth and sporulation of *Streptomyces venezuelae* was between 5.5 and 11.5. They stated that the pH of the media strongly influenced the acid tolerance of the *Streptomyces* species.

To achieve the highest production rate, *Streptomyces venezuelae* should be grown in media with the pH maintained between 7 and 7.5 (Dupius, 2010; Burdock et al., 2008; Jakeman et al., 2006; Doull et al., 1991). Doull et al. (1991) studied the effects of varying pH (6, 6.5, 7 and 7.5) on jadomycin production and found that the highest jadomycin yield was

obtained in media with a pH of 7-7.5. Negligible amount were obtained when the pH was between 6 and 6.5.

Thakur et al. (2009) examined the effect of acidic and alkaline pH on antibiotic production by *Streptomyces sp.* and reported poor growth when the initial pH was adjusted between 5 and 6. However, the maximum biosynthesis of antimicrobial agent and cell growth were observed at a pH of 7.5.

Antonopoulos et al. (2001) examined the effect of pH on *Streptomyces albus* enzyme activity within the pH range 6 - 11.5 and found that maximum enzyme activity was obtained at a pH of 6.5 - 7. The enzyme activity within this pH range was maintained at 90%.

Bhattacharyya et al. (1997) reported that a pH below 7 affected *Streptomyces hygroscopicus* cell growth and antibiotic production and concluded that pH 7 was the optimum for antibiotic yield. James et al. (1991) stated that pH range between 6.5 and 7 resulted in good growth and antibiotic (granaticin) production for *S. thermoviolaceus*.

Osman et al. (1991) reported that antibiotic production from *Streptomyces* isolates from Egyptian soil was favoured at a pH of 7.5. Reducing or increasing the pH level below or above this pH resulted in poor antibiotic yield and cell growth.

To buffer the pH to the near neutral range, MOPS is commonly used for *Streptomyces* species (Kendrick et al., 1982; Jakeman et al., 2006). Jakeman et al. (2006) reported that using MOPS buffer (10 mM) was sufficient to maintain pH and increasing the amount of MOPS inhibited the jadomycin yield. One pH unit drop was noted after 24 hours, but remained constant for next 48 hours making it more significant.

Glazebrook et al. (1990) reported that MOPS were able to maintain the pH of the production medium with every carbon or nitrogen source. MOPS buffer has a logarithmic acid dissociation constant (pKa) of 7.2, making it suitable for buffering solution at near neutral pH.

Jakeman et al. (2006) and Burdock et al. (2008) used MOPS in the production media for jadomycin B production and found that MOPS was sufficient to maintain the pH of both

growth (MYM growth media) and production media (MSM production media) at near neutral values for every carbon and nitrogen source used.

3.3.5. *Phage Infection*

Doull et al. (1993) examined phage infection and used transducing phage SV1 virus isolated from soil (which is virulent and temperate in nature) to cause secondary metabolite production. Adding *Streptomyces venezuelae* spore suspension to the galactose (carbon source) and isoleucine (nitrogen source) rich production medium resulted in the formation of orange pigments after 48h at 30°C. Increased cell lysis and pigmentation was reported after the phage SV1 infection of mycelium cells on the solid medium.

Martinez and Olivares (1979) examined the ability of phage-sensitive and phage immune clones isolated from original strains of *Pseudomonas reptilivora* to yield antimicrobial compounds in a defined culture medium in the presence of temperature-sensitive temperate phage (*Pseudomonas* Φ6). The loss of antibiotic production in phage sensitive cells was noted whereas high production activity was seen in phage immune cells. The sub-culturing and inhibition of lysogenic conversion by temperature-sensitive temperate phage (*Pseudomonas* Φ6) in phage sensitive cells resulted in poor production. They reported that spontaneous or artificial induction of the temperate phage at normal culture temperature (28°C) results in antibiotic production.

Vešligaj et al. (1981) examined the antibacterial production of *Streptomyces rimosus* infected by RP1 actinophage and uninfected *Streptomyces rimosus* cultures. The RP1 infection of the host cell resulted in a dramatic inhibition in viable cell growth and antibiotic production (oxytetracycline). Characterization of the mutant phage indicated that phage RP1 had no influence on their antibiotic production or cell growth. Since these strains differed from the wild type in RP1, they were considered as semi-resistant mutants.

3.4. *Jadomycin Application*

Jadomycin belong to the angucyclinepolyketide group which represents the largest class of secondary metabolite products in nature. They have exhibited various biological activities.

The cytotoxic effects of the jadomycin analogues against various cancer cell lines are shown in Table 3.3 and against breast cancer cell line (MDA-MB-435) are shown in Figure 3.4.

Zheng et al (2005) reported five of designated jadomycin B (Ala, F, V, S and T) with incorporated amino acids isoleucine (B), alanine (ala), valine (V), phenylalanine (F), serine (S) and threonine (T). These diastereomeric jadomycin mixtures consist of 3a-S and 3a-R configuration. The ability of various amino acids to incorporate in the jadomycin skeleton shows that this reaction occurs non-enzymatically. The author stated that all derivatives of jadomycin showed improved cytotoxic effects against human tumor cell lines (cancer cells). The oxazolone ring with its side chain derived from the different derivatives has an important effect on biological activity. The study shows that Jadomycin Ala was less active compared with other derivatives, while serine derived Jadomycin S had most significant impact against the cancer cell lines. This is because the derivative from alanine has a simple methyl group attached to oxazolone ring while the derivative from serine has a hydroxyl at the oxazolone ring instead of the methyl group.

The cytotoxic study conducted by Borrissow et al. (2007) on 19 different types of jadomycin derivatives against breast cancer cell lines (T-47D and MDA-MB-435) showed significant effects against the cells. Jadomycin H was the most active when compared with other derivatives especially on the MDA-MB-435 cell. The study observed that derivative with small polar side chains in the oxazolone ring exhibited much greater level of cytotoxic action against cancer cell lines. The overall study indicates that the more active compounds were those with hydroxymethylene side chains and the least active were those with aromatic side chains.

The antimicrobial activities of these jadomycin compounds were examined by Jakeman et al. (2009) by screening against several gram–positive and gram–negative bacterial strains (*S. aureus, S. epidermidis, Pseudomonas aeruginosa, Enterococcus faecalis* and *Bacillus subtilis*). Greater activity was noted in gram-positive microbes than gram-negative. It showed that structural analogues of PKS-derived natural products provide an important range of biological activity. The study showed that structure and substitution around oxazolone ring

Table 3.3. Cytotoxic effects of different types of jadomycin against various cancer cell lines.

Jadomycin Type	Cancer Cells	Description	Reference
B, ala, F, V, S, and T	Human tumor cell lines (lung and blood): (HepG2, IM-9, IM-9/Bcl-2 and H460)	Jadomycin S exhibits significant prevention against HepG2, IM-9 and IM-9/Bcl-2, while F against H460. Result shows that side chains of the oxazolone ring formed from incorporated amino acids cause significant impact.	Zheng et al (2005)
B,V,T,S,F,Y,S-Phe,M,H,W,N,G,DV,DT,DM,R-Phe,β-Ala,T	Breast cancer cell lines: T-47D and MDA-MB-435	Compounds were consistently more effective against cancer cell lines, jadomycin H and B two fold higher potency against T-47D cells, Jadomycin S, DT and T effective against both while Y,H and β-Ala least potency.	Borrissow et al (2007)
B,S,T	Human cancer cell lines (A549, HeLa, and MCF-7)	Jadomycin S and T prevented the growth of all human cancer cell lines, although the rate of inhibition was less compared to Jadomycin B. Identification of new aurora-B inhibitor (Jadomycin B) through biochemical approaches were identified.	Fu et al (2008)
B,L,F,N and G	Human breast cancer and a melanoma cell line (T-47D and MDA-MB-435)	Structural analogues of PKS-derived natural products provide vital biological activity. Both Jadomycin B and L contains aliphatic side chains extending from oxazolone ring α-carbon and Jadomycin F having benzyl group connecting to oxazolone ring α-carbon exhibited inhibition to cancer cell lines.	Jakeman et al (2009)
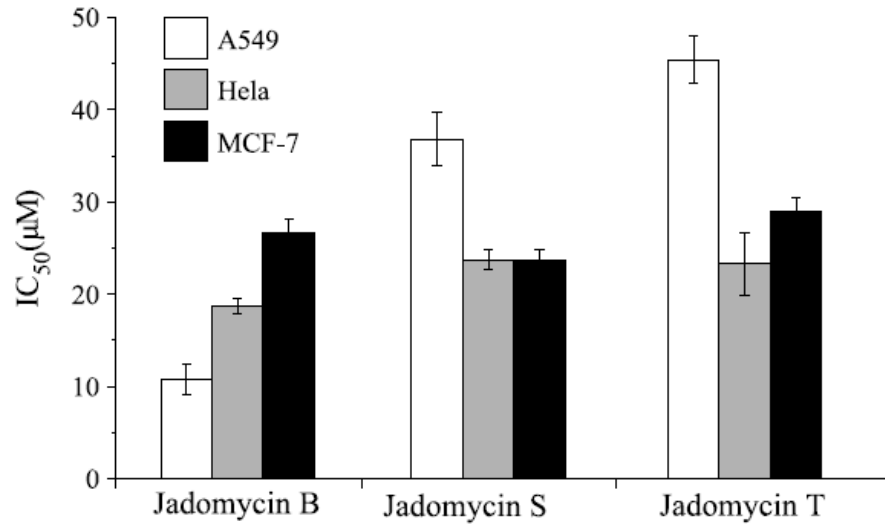
(a) Human cancer cell lines (A549, HeLa, and MCF-7 cells) exposure to increasing concentration of jadomycin B, S and T for 48 hours to measure IC50 values.
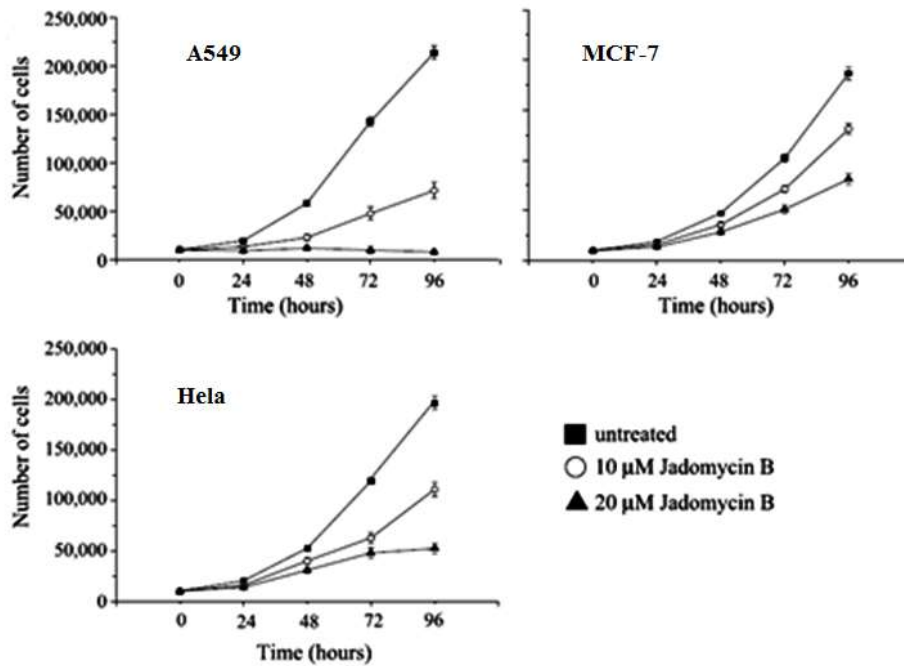
(b) Human cancer cell lines treated with different jadomycin B concnetration for 96 hours and number of cells were noted.

Figure 3.4. Inhibiting the growth of several human cancer cell lines in dose dependent manner (Fu et al., 2008).

tends to confer distinctive antimicrobial activities on jadomycin analogues and gives the impetus to explore the unique biosynthetic mechanism involved during jadomycin production. In respect to antimicrobial activity of jadomycin derivatives, jadomycin B and L (which contain aliphatic side chains extending from the oxazolone ring α-carbon) and jadomycin F (which contains a benzyl group attached to oxazolone ring α-carbon) were the most significant against the cancer cells. Distinguishable order of antimicrobial activity is due to the distinctive nature of bacteria toward the compounds, possibly due to the different mechanisms of action or various modes of affinities for their biological targets. Research related to their mode ofaction, specificity of the jadomycin and cellular toxicity is on-going (Jakeman et al., 2009).

3.5. *Streptomyces venezuelae*

Streptomyces (Genus – Actinomycetes) is found widely in nature and it is the largest producer of secondary metabolites. Actinomycetes grow especially in soil environments and produce antibiotics for survival (Hopwood, 2007; Taddei et al., 2006). These antibiotic substances can act against other bacteria and fungus and some compounds also exhibit effective biological significance as anti-cancer metabolites (Dupuis et al., 2011; Izard, 2001; Jakeman et al., 2005:2006). Scanning electron images of *Streptomyces* are shown in Figure 3.5.

Some of the antibiotics produced by the *Streptomyces* genus are shown in Table 3.4 (Sharma, 2000). *Streptomyces venezuelae* is known to produce at least two secondary metabolites (chloramphenicol and jadomycin) during its growth cycle in production media (Burdock et al., 2011; Bhatnag et al., 1988; Gottlieb et al. 1954). *Streptomyces venezuelae* exhibit growth stages comprising of extensive elongation and branching to form vegetative mycelia, aerial hyphae and spore formation similar to filamentous fungi. The cell walls of vegetative growth (Figure 3.6) are structurally and chemically similar to other known type of gram-positive bacteria (Bradley and Ritz, 1968; Flardh, 2003; Classen et al., 2006). The microbial sporulation is linked to environmental stress factors or exhaustion of nutrients availability. In spite of their characteristic similarity to fungi, the cell walls during vegetative growth are identical to other gram positive bacteria (Bradley and Ritzi, 1968; Kieser et al., 2000).
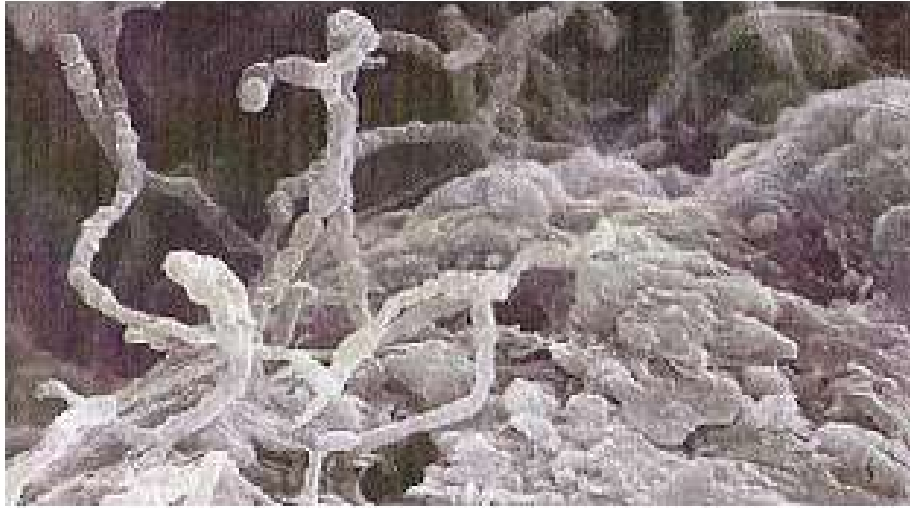
(a) Scanning electron image of *Streptomyces* in growth medium (Hopwood, 2007)
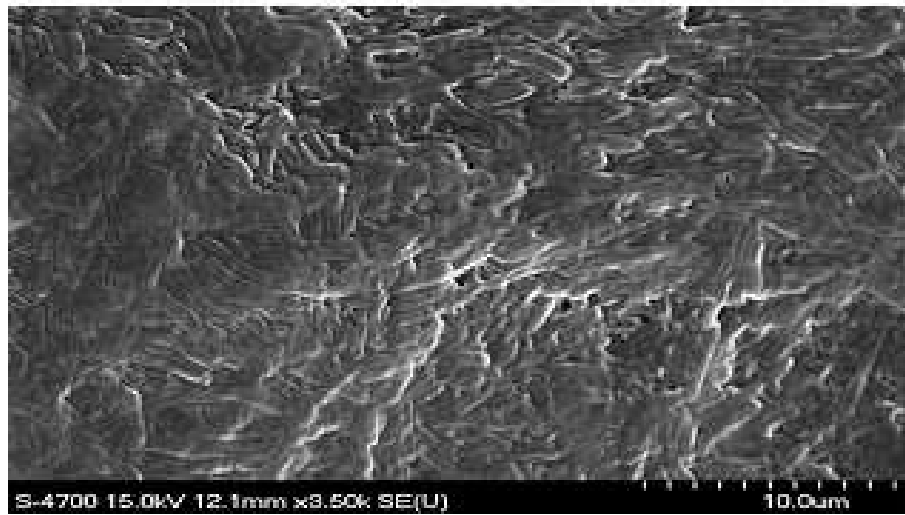
(b) Scanning electron microscopic image of *Streptomyces* venezuelae during
   growth in MYM broth (Brooks et al., 2012)

Figure 3.5. Scanning electron microscopic images of *Streptomyces venezuelae*.

Table 3.4. Common antibiotics produced by *Streptomyces* genus (Sharma, 2000).

Antibiotic	Bacterial Producer	Antibiotic Spectrum
Streptomycin	*S. griseus*	Most Gram-negative bacteria
Spectinomycin	*Streptomyces spp.*	*Mycobacterium tuberculosis*, *Neisseria gonorrhoeae*
Neomycin	*S. fradiae*	Broad spectrum
Tetracycline	*S. aureofaciens,* *S. rimosus*	Broad spectrum
Chlortetracycline	*S. aureofaciens*	Broad spectrum
Erythromycin	*S. erthreus*	Often used in place of penicillin
Lincomycin	*S. lincolnesis*	Obligate anaerobes
Nystatin	*S. noursei*	Antifungal
Chloramphenicol	*S. venezuelae*	Broad spectrum
Amphotericin	*S. nodosus*	Antifungal
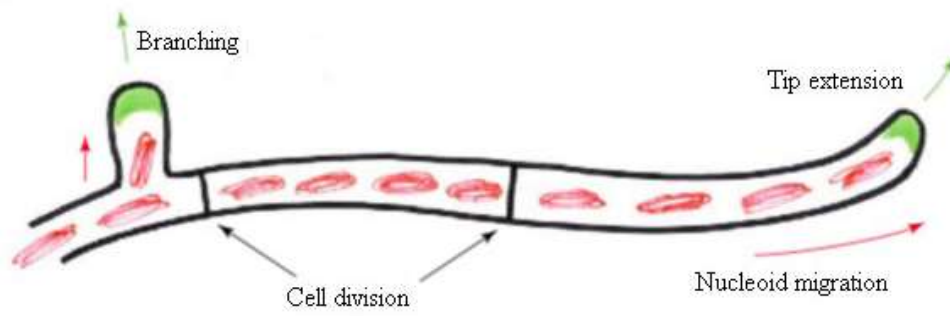
Figure 3.6. Image of *Streptomyces* growth illustrating its tip extension and branching mechanism (Flärdh, 2003).

According to Kieser et al. (2000) *Streptomyces venezuelae* is one of the few *Streptomyces* species that have the capacity to sporulate in liquid media, thereby favoring the production of secondary metabolite during the stage of sporulation. The secondary metabolite production in *Streptomyces venezuelae* takes place normally during the transition from vegetative growth to spore as a result of stress or competition in environment (Brooks et al., 2012; Hopwood, 2007). Secondary metabolites are produced when the nutrients required for the primary metabolism are limited or as a result of the morphological alteration or change in the intermediate structure that occurs in *Streptomyces* when the filamentous mycelium form spores (Shapiro, 1989; Vining and Stuttard, 1995).

Most *Streptomyces* species are mesophillic bacteria and can tolerate temperatures between 15-40˚C. Some other types like *S. thermophiles, S. thermofuscus, S. casei, S.thermodiastiticus* can survive temperatures equal to or greater than 50ºC, exhibiting most of their activity in compost and manure piles where the temperature exceeds 40˚C (Sharma, 2000).

3.5.1. *Life Cycle*

The life cycle of *Streptomyces* bacteria starts with the germination of a single spore which produces more multi-nucleoid filaments. The overall view of the *Streptomyces* life cycle is shown in Figure 3.7. This structure then branches and elongates on the upper surface and into the culture medium in order to form vegetative mycelium. Most of their activities are in compost and manure piles where the temperature exceeds 40˚C (Sharma, 2000). The hyphae, a complex network of filaments, will penetrate into the medium, thereby using the available nutrients with the help of hydrolytic enzymes. These motility characteristics of the *Streptomyces* vegetative filaments give a big advantage over other bacteria of less motility when it comes to colonizing solid substrates in the soil.

The depletion of the nutrient supply in the surrounding environment results in the breakdown of substrate mycelium and the formation of aerial hyphae. This aerial growth corresponds with the start of secondary metabolism in the cultures grown on solid media. When the aerial hyphae extension is stopped, their mutigenomic tips undergo multiple
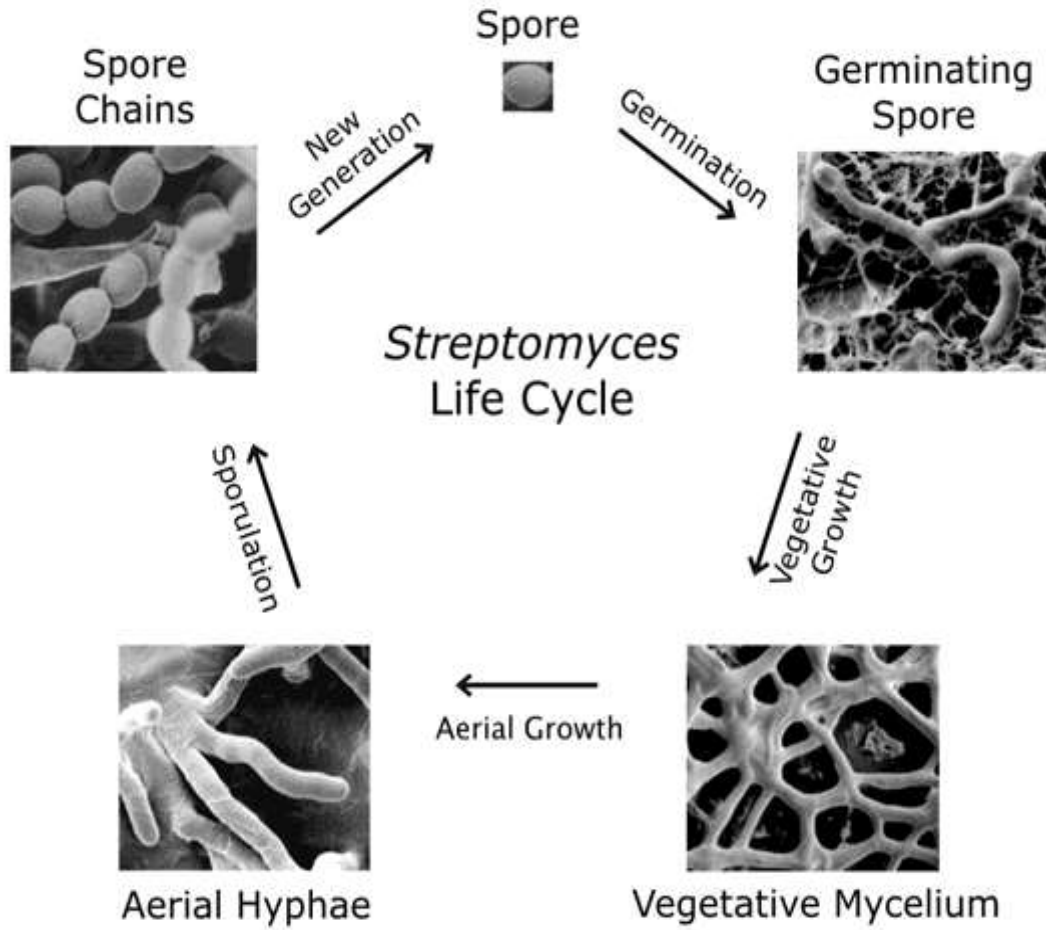
Figure 3.7. Life cycle of *Streptomyces* (Kieser et al, 2000).

septation to form unigenomic prespore compartments. These mature spores are held together in the form of chains (about 50) and later develop a grey pigment. Dissimilar to endospores, which belong to Gram-positive bacteria (*Clostridium* and *Bacillus*), the *Streptomyces* exospores are not resistant to the extreme pH or heat and are less dormant. But they seem to be fairly resistant to desiccation (Kieser et al., 2000). The growth of *Streptomyces* is aerobic with both the substrate and aerial mycelia producing an odor characeristic of soil (Taddei et al., 2006). During their initial growth on solid media rich in nutrients, *Streptomyces* colonies have a smooth surface.The aerial mycelia can appear to be powdery or grainy thus indicating defined colony surfaces (Sharma, 2000). A wide range of the spore, mycelia and the substrate colors are observed in this bacterial genus. Figure 3.8 shows the transition from filamentous vegetative mycelium to sporulating hyphae.

3.5.2. *Structural Characteristics*

The primary structure of *Streptomyces* is formed by the loose system of hyphae and involves progressive growth mainly distributing on the substrate surface (Sharma, 2000). Predominant colors of the substrate mycelia are yellow, beige and ivory with violet-purple and red-violet (Taddei et al., 2006). During the vegetative growth, *Streptomyces venezuelae* gives out the colorless hyphae in single –footed branches of about 0.9 – 1.8 μm in diameter (Waksman, 1957). The aerial mycelium which grows in the more densely packed structural form (than the substrate mycelia) move into the air (Sharma, 2000). The colors found in the aerial structures are gray, red, white, yellow, blue, with green and black (Taddei et al., 2006). The aerial mycelia appear light tan, gray or light pink when observed without magnification. Their appearance gives a lavender color when seen through the microscope. They appear to be slightly curved or straight and are generally similar to the vegetative form. The aerial mycelia are larger and less branched than the vegetative mycelia, having a thickness of about 5 nm in the outermost layer thereby forming thick sheath (Bradley and Ritzi, 1968). Tables 3.5 and 3.6 show the color characteristics of *Streptomyces* species.

After formation of the aerial hyphae, chains of spores develop forming along the end of the aerial mycelia. Their sporulation has been extensively analyzed in the *S.coelicolor* and found to have four stages .The first stage involves in the coiling of the aerial mycelium
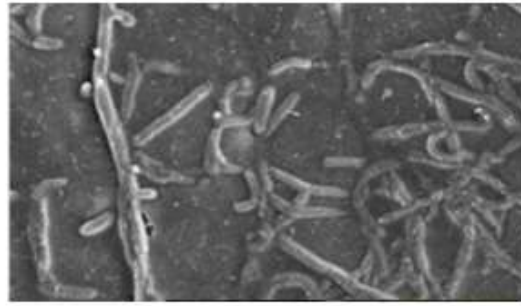
(a) Filamentous vegetative mycelium
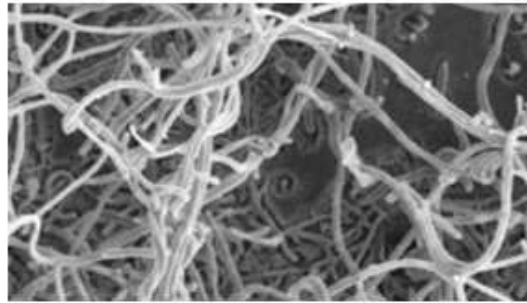
(b) Aerial hyphae
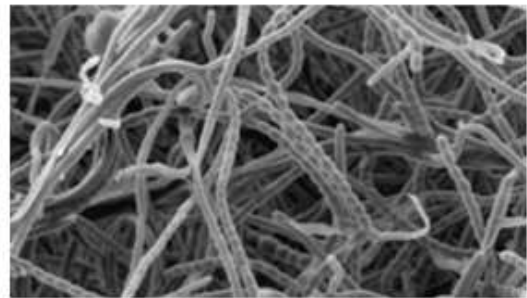
(c) Sporulating hyphae

Figure 3.8. Scanning electron microscopic image describing the transition of *S.venezuelae* from filamentous vegetative mycelium to sporulating hyphae (Claessen et al., 2006).

Table 3.5. Characteristic substrate mycelium colour in *Streptomyces* spp. (Sharma, 2000).

Characteristic Pigment of Substrate Mycelium	Species
Orange turning to red	*S. aurantiacus*
Red turning to blue	*S. californicus*
Red turning to violet	*S. coelicolor*
	S. cyanaus
Green turning to olive	*S. olivoviridans*
Grey turning to black	*S. hygroscopicus*

Table 3.6. Pigment formation in *Streptomyces* spp. (Sharma, 2000).

Characteristic Pigment	*Species*
None	*S. albus*
Faint brown	*S. longisporus*
	S. rochei
Blue	*S. coelicolor*
	S. pluricolor
	S. cyaneus
	S. violaceus
Green changing to brown	*S. verne*
	S. viridans
Yellow or golden yellow	*S. flaveolus*
	S. parvus
	S. rimosus
	S. aureofaciens
	S. xanthophaeus

followed by the growth of the double annulus. The double annulus is continuous with the cell wall and membrane structures in order to form a partition between the spores. Finally septa formation is seen with the thick cell wall being laid down. The morphological change of the spore from cylindrical shape to the ellipsoidal is seen finally (Sharma, 2000). Taddei et al. (2006) reported that the growth of *S.venezuelae* spores is straight and flexuous chains and appear as tightly twisted or coiled shorter chains. Waksman (1957) states that *S.venezuelae* spores are oblong or ellipsoidal in structure measuring about 0.4 - 0.8 by 0.7 - 1.6 μm as shown in *Streptomyces coelicolor* (Figure 3.9). The surface of *S.venezuelae* spores appears to exhibit a mosaic pattern. These patterns are also observed in the other species of *Streptomyces* genus. The cell wall structure enclosing the spores has been found to be approximately 30 nm thick (Dietz and Mathews, 1962).

Van Iterson (1965) reported that upon exoposure to nutrient broth, the spores expand widely to form a membranous system. During their germination, spore ribosomes form which measure about 12 nm in diameter. Bradley and Ritz (1968) extracted the proteins from the *S.venezuelae* mycelia and spores in order to determine the amino acid composition. They found that the amino acid composition (arginine and the leucine) were measurable in vegetative mycelium whereas the spores contained less amount of the amino acids.

3.5.3. *Production of Metabolites*

Two known metabolites are produced from the soil bacteria *Streptomyces venezuelae* namely jadomycin and chloramphenicol. Jadomycin is produced when *Streptomyces venezuelae* is subjected to nutrient deprived environmental conditions or exposed to environmental shocks.

Chloramphenicol: The production of chloramphenicol by *Streptomyces venezuelae* was first discovered in 1947 and was applied clinically for several years and then ceased after the primary metabolite showed adverse effects such as aplastic anemia (Sheridan et al., 2008). Chloramphenicol is a broad range antibiotic which is bacteriostatic in nature and inhibits the translation step of the ribosome function as its mode of action (Chang, 1999; Izard, 2001; Sharma, 2000).Chloramphenicol inhibits the peptide bonds formation by obstructing the
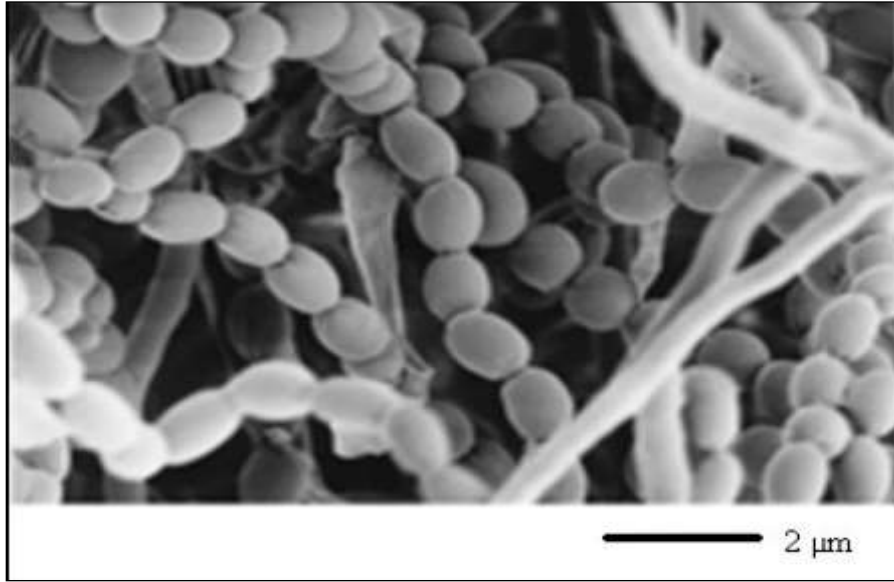
Figure 3.9. Oblong or ellipsoidal *Streptomyces coelicolor* hyphae after sporulation (Ryding et al., 1999).

enzyme named peptidyltransferase present in the 50S prokaryotic ribosomal subunit (Chang, 1999; Izard, 2001).

Chloramphenicol (D-(-)threo-2-dichloracetamido-1-p-nitrophenyl-1,3-propanediol) is a relatively simple structural metabolite and was one of the first antibiotics to be produced synthetically. This metabolite inhibits bacterial proteins by directly preventing substrate binding during synthesis. Lower cost chemical syntheses were made possible by its simple chemical structure. By production of the enzyme called chloramphenicol phosphotransferases, *Streptomyces venezuelae* are able to prevent the toxic impact produced by the chloramphenicol. This enzyme phosphorylates the initial (C-3) hydroxyl on the chloramphenicol (Mosher et al., 1995).

Gottlieb et al. (1954) noted that chloramphenicol production starts to occur within 24hrs after inoculation and reached a peak after 60 hours. The studies also concluded that compounds similar to chloramphenicol (such as phenylserinol and phenylserine) had significant effects and are likely to increase the primary antibiotic production. The authors concluded that the physiology of *Streptomyces venezuelae* did not change during bacterial growth and secondary metabolite production.

Bhatnagar et al. (1988) reported that during the production of chloramphenicol, changes in availability of carbon sources or nitrogen sources had an adverse effect on production, whereas the specific rate of primary metabolite are insensitive to excess or lower nutrient sources.

Jadomycin: The jadomycins show promising activity against cancer cell lines as aurora kinase inhibitors. The production of jadomycin was initially linked to growth at high temperatures (Ayer et al., 1991; Jakeman et al., 2006).

The jadomycins are named according to the type of side chain attached to their structure due to amino acid present in the production media. The aurora kinase inhibitors function by inhibiting the phosphorylation of histone in a dose-dependent manner. They prevent the aurora-B kinase activity by competing for ATP (adenosine triphosphate) in the kinase domain. This causes apoptosis in tumor cells without negative reactions (Fu et al., 2008).

The production of jadomycins are related to the heat shock response protein of *Streptomyces venezuelae*, where an increase in temperature can trigger cellular responses which result in the expression of specific heat shock genes (Doull et al., 1994; Ayer et al., 1991). An other means of inducing the stress is with alcohol (ethanol), where it has been reported to be more powerful and effective for antibiotic production than heat stress techniques, thereby making it more favorable for laboratory scale production (Singh, 1992). Growth media (mainly Malt-extract, Yeast-extract, Maltose (MYM) media) populated with healthy numbers of vegetative cells are transferred to MSM production media where stress is induced to initiate antibiotic production. In *Streptomyces venezuelae*, genes specifying the production of jadomycin appear to be fully expressed only if their growth are controlled and restricted by depletion of particular nutrient or induction of several stress conditions (Jakeman et al., 2006; Demain et al., 1981; Vining, 1986). Therefore, controlling the potential growth of bacteria in the media promotes the production of jadomycin metabolite.

3.6. *Monitoring Microbial Growth Techniques*

The rate of microbial growth population can be determined during fermentations by various techniques such as (a) gravimetric technique (b) direct microscopic determination (c) plate count using MYM agar medium (d) direct absorbance measurements and (e) measuring the cell components. However, these methods tend to have some drawbacks which make it difficult to analyse the growth. Table 3.7 summarizes different techniques used to estimate the cell concentration and their disadvantages.

All these methods are not accurate and lead to some error, (since the suspension contains both live and dead bacteria) and require intensive labor and more time for preparation (Mahmoud, 2005).

Plate counting requires a period of 24-72 h for bacterial growth on solid agar plates. This method often results in extensive bacterial growth and inability to count larger populations thereby underestimating the number of viable colonies by the order of one magnitude resulting in inaccuracy (Buck, 1979; Daley, 1979). However, this method is inexpensive and

Table 3.7. Summary of different techniques to estimate the cell concentration and their disadvantages (Kennedy et al., 1992).

Strategy	Disadvantages
Direct microscopic examination	labor intensive, cells adhering to solid surface causes problems
Colony counting on a plate	requires considerable time, numerous cells adhering toone solid particle can lead to one colony
Prior separation of cells and solid	complete separation is very difficult
Preferential dissolution of the solid	difficult to find reagent that will only dissolve solids
Preferential dissolution of the cells	difficult to find reagent that will only dissolve cells
Dissolution of cells and solids followed by a separation technique	difficult to find a property in which the solubilized cells and solids differ
Measurement of a selective component that is not present in the solid	difficult to find a substance present in cells but not in the solid
	most metabolites are not a constant proportion of the cell mass during all stages of development
Metabolic measurements	metabolite production proportionality to cell concentration may vary with time during the fermentation
Measurement of a physical property	very few physical properties effectively distinguish between cells and solids
	shows potential for further development
Deconvolution of light-based data	effect of gas bubbles and cell morphology on the technique has not been investigated shows potential for further development

determines the population of active live bacteria (Tabacchioni et al., 2000; Trevors, 199b; Dix and Webster, 1995). This method is sufficient for counting unicellular organisms but does not give detailed information of the extent of *Streptomyces* growth. Since a microbial colony starts from spores or from fragments of mycelium, it is necessary to estimate the number of colonies appearing on plates. Brooks et al. (2011) showed the that rate of viable count of *Streptomyces venezuelae* increased steadily with the shaking incubation time. This may be caused by either gradual separation of particles disintegrating or aggressive breaking of mycelium.

Optical density measurements require little time, but the inability of this technique to differentiate between live and dead cells results in errors (Mahmoud and Ghaly, 2004). Wei et al. (1983) measured the cell concentration of *Streptomyces cerevisiae* in semi solidified gelation solution (where the gelation and other media components did not produce interfering absorbance) and stated that this was not successful in other semi solid media. Hong et al. (1987) stated that this technique is only applicable if the solids content do not change during the fermentation and is much less than the live cells. During the secondary metabolite production, the solid concentrations tend to change significantly as ions in the media and absorb the light strongly. The authors concluded that this technique cannot be used to distinguish between the live and dead cells.

Physically determining the total number of cells under a microscope is labour intensive and time consuming. Dyeing the cells and proper dilution methods make it difficult and can hinder the accuracy of this technique. Depending upon the type of dyeing method, the live cells may be killed or made unsusceptible for analysis. With the enumeration under the microscope, foreign debris may often be mistaken as cells resulting in major errors (Kennedy et al., 1992).

There are other indirect techniques such as (a) ATP determination (b) Glycosamine determination (c) Extracellular laccase analysis (d) CO_2 and O_2 activity of the cell determination. However, the techniques that involve analysing cell constituents are cost ineffective (Lonsane et al., 1985).

3.7. *Dehydrogenase Activity*
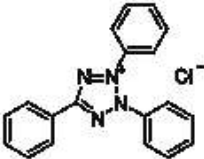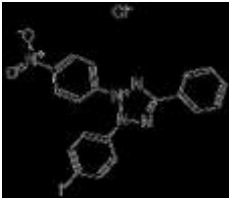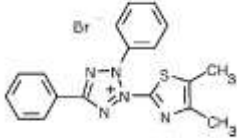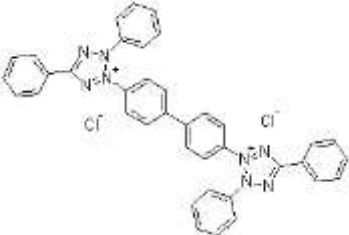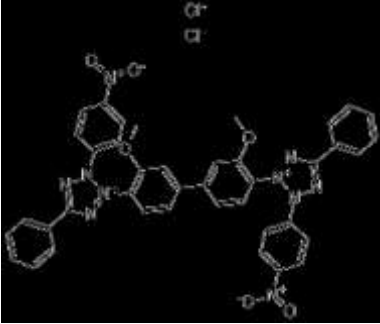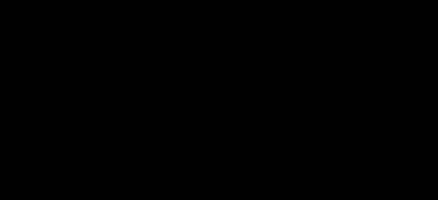
The dehydrogenase activity assay which employs dye (tetrazolium salts) as an electron acceptor to measure the bacteria, is a more accurate, cost effective, time efficient and simpler way to determine the bacterial biomass during the fermentation process (Ghaly and Mahmoud, 2006). Various tetrazolium salts have been used and proved to be effective in measuring the bacterial viability (Table 3.8). This technique is based on the fact that all living cells produce dehydrogenase enzymes. These enzymes in living cells catalyze the oxidation process of organic material by transferring electrons and hydrogen through a series of electron carriers to oxygen (an electron acceptor) to form water molecules (Chander and Brookes, 1991; Roger and Li, 1985; Ruhling and Tyler, 1973; Ghaly et al., 1989).

The dye used in the dehydrogenase activity assay comprises of tetrazolium salts which are stable and water soluble (Ghaly and Mahmoud, 2006). These salts are available from plain white to less bright yellow and brown. The advantages in using these dyes are: (a) they have the ability to form highly coloured compounds upon reduction (formazan) (b) they are non-toxic and non-autoxidizable and (c) no complex mechanisms involved and are easily reduced (Altman, 1976).

Intracellular dehydrogenase relates to oxidoreductases and initiates the oxidation mechanism of organic compounds by dividing the two hydrogen atoms. Many particular dehydrogenase transfer hydrogen atoms to either nicotinamide adenine dinucleotide or nicotinamideadenine dinucleotide phosphate. Then, these hydrogen atoms participate in a reductive process of biosynthesis with the help of these co-enzymes (Mersi and Schinner, 1990).

The general view of the dehydrogenase process involves the various dehydrogenases which are part of the fundamental enzyme network of all microorgansims (enzymes of citrate cycle, N metabolism and respiratory metabolism). The hydrogen atoms are then transferred to soluble tetrazolium salts resulting in red formazan. These are then determined calorimetrically after extraction with a solvent. The overall dehydrogenase mechanism is described as follows (Mahmoud, 2005):

Table 3.8. Commonly used Tetrazolium salts.

Systematic Name	Empirical Formula	Structure	References
2,3,5-triphenyltetrazolium chloride (TTC)	$C_{19}H_{15}ClN_4$		(Burdock et al., 2010); Mahmoud and Ghaly, 2004)
2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT)	$C_{19}H_{13}ClIN_5O_2$		(Friedel et al., 1994); Gong, 1997)
(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT)	$C_{18}H_{16}BrN_5S$		(Van Ophem et al., 1991; (Stentelaire et al., 2001)
3,3'-(4,4'-Biphenylene)bis(2,5-diphenyl-2H-tetrazolium chloride)	$C_{38}H_{28}Cl_2N_8$		(De Jong et al., 2001)
3,3'-(3,3'-dimethoxy-4,4'-biphenylylene)-bis-[2-(p-nitrophenyl)-5-phenyl-2h-tetrazollium chloride)	$C_{40}H_{30}Cl_2N_{10}O_6$		(Saito, 1993; (Chikamori et al., 1998)
3,3′-(3,3′-Dimethoxy-4,4′-biphenylene)bis[2,5-bis(p-nitrophenyl)-2H-tetrazolium chloride	$C_{40}H_{28}Cl_2N_{12}O_{10}$		(Selyakh et al., 1990)

$$RH_2 \xrightarrow{\text{Dehydrogenase}} R + 2H^+ + 2e^-$$

Substrate → Oxidized Substrate (1)

$$2H^+ + \left[C_6H_5C \begin{matrix} N\text{-}N\text{-}C_6H_5 \\ | \\ N\text{=}N\text{-}C_6H_5 \\ | \\ Cl \end{matrix} \right]^- \longrightarrow HCl + C_6H_5C \begin{matrix} N\text{-}NH\text{-}C_6H_5 \\ \\ N\text{=}N\text{-}C_6H_5 \end{matrix}$$ (2)

Triphenyl Tetrazolium Chloride (TTC)

Triphenyl Formazan (TF)
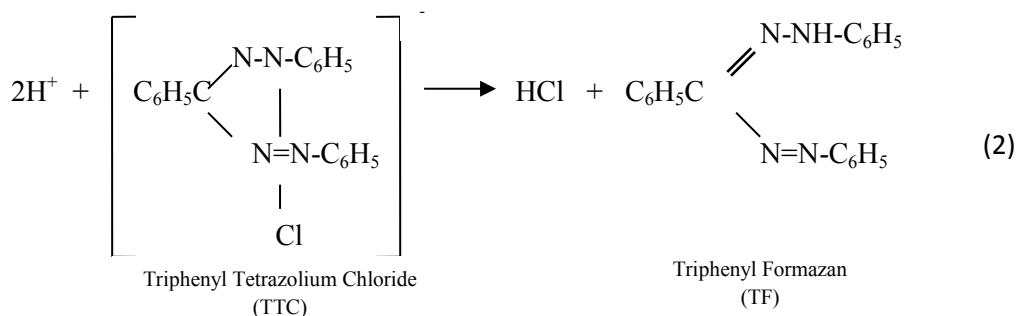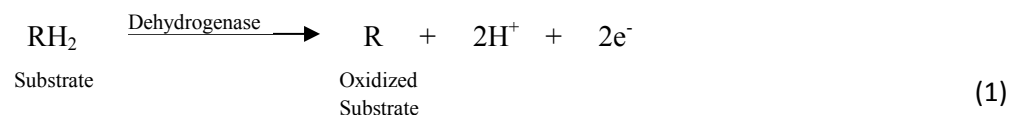
The procedure to use these salts to measure the dehydrogenase activity requires optimum incubation time, temperature, extraction solvent and pH. This technique requires incubating the sample in the presence of triphenyl tetrazolium chloride (TTC) and an electron donating substrate. The technique includes estimating the oxidation of organic material with dehydrogenase enzymes which leads to hydrogen atom production (Mahmoud and Ghaly, 2004; Burdock et al., 2011). The tetrazolium salt which is a hydrogen acceptor is employed after the formation, thereby associating the substrate oxidation process with tetrazolium salt reduction and resulting in a coloured dye or formazan product. This dye is then extracted from cells using ethanol or methanol. The colour intensity of the extracted dye is then measured using a spectrophotometer (Griebe et al., 1997; Mahmoud and Ghaly, 2004).

Many studies have shown dehydrogenase activity measurement to be effective and efficient in measuring the bacterial metabolic reactions in soils (Lenhard, 1956; Klein et al., 1971; Casida, 1977; Chander and Brookes, 1991; Friedel et al., 1994; Gong, 1997), sludges and in swine manure. Tango and Ghaly (1999) and Ghaly and El-Taweel (1995) employed this technique during the production of alcohol, during lactic acid fermentation from cheese whey. This technique is also employed in seed viability enumeration (Vujanovic et al., 2000) and for fungal spore viability assessment (Stentelaire et al., 2001).

3.8. *Factors Affecting the TTC Reduction*

The principle application of the dehydrogenase activity in causing reduction from TTC to

red, stable, non-diffusible triphenyl formazans in live microbial cells has been demonstrated (Beloti et al., 1999). Many research studies show that TTC can be used for quantitative measurements of viability of bacterial live cells, but specific techniques need to be worked for each type of species. Factors like temperature, pH, incubation time period and proper solvent for extracting the triphenyl formazans must be considered for a reliable procedure (Beloti et al., 1999).

3.8.1. *Bacterial Species*

Huddleson and Baltzer (1950) stated that different microbial colonies or types of the same species may develop several shades of red when subjected to TTC. Gershenfield and Weber (1951) reported that coagulase-positive *Staphylococcus* species would result in orange colored colonies whereas coagulase-negative *Staphylococcus* species would form pink colored colonies when TTC was added to the growth medium. Turner et al (1963) observed that *Streptococcus cremoris* could be distinguished from *Streptococcus* lactis by means of TTC. This was successful because the ability of one species to reduce the dye while the other cannot. Rioux et al. (1960) showed that *Candida albicans* could be differentiated from other yeast, because they would yield white colored colonies while *C. albicans* would yield pink colonies.

Ghaly and Mahmoud (2006) used a simple and fast method to determine biomass quantity during solid state fermentation of shrimp shells using *Aspergillus niger.* The study indicated that the TTC salt was able to penetrate the cell plasma membrane of *A.niger* spores and significant amount of colored compounds could be extracted.

Burdock et al. (2011) used the dehydrogenase activity test to measure the quantity of live cells and to determine the optimal stress time of *S.venezuelae* for jadomycin production. The colourless TTC was reduced to a red water insoluble compound, triphenyl formazan (TF). The TF was retained within the cells and resulted in highly colored colonies upon their growth in MYM agar. Brooks et al. (2012) used the dehydrogenase test as a potential tool for the determination of *S.venezuelae* growth and metabolism during the secondary metabolite production.

3.8.2. *pH*

The reduction of TTC is more intense at higher pH for lichens which is indicated by a strong color change (red color). Backor and Fahselt (2005) reported on the ability of TTC to measure lichens and their bionts. The TTC reduction took place when the pH was 1.5 where no reduction was seen at higher pH. *Flavoparmelia caperata* lichen was more sensitive than *Cetrariaarenaria* and caused TTC reduction at pH 3.

The study conducted by Ghaly and Mahmoud (2004) showed that higher pH conversion of TTC occurs chemically but not enzymatically. The experiment was also done in acidic environment in cheese whey and the results showed no reduction of TTC with pH less than 7.

Shuler and Kargi (1992) reported that variation in pH may result in hampering the enzymes by: (a) altering the ionic form of the active sites in enzymes thereby affecting its reaction rate and activity, (b) affecting the affinity of the substrate to enzyme and (c) altering the three dimensional shape of these enzymes.

Ghaly and Ben-Hassan (1993) found the optimum pH value for the dehydrogenase activities of *Kluyveromyces fragilis* and *Candida Pseudotropicalis* using TTC was 7. They reported that pH variation of 3 units tends to inhibit enzyme activity within these organisms.

Ghaly and Mahmoud (2006) examined the effect of pH on dehydrogenase activity of *Aspergillus niger*. The results showed that the reduction of TTC to TF was not significant under acidic conditions (< 6.5). They noted TTC reduction at pH values within the range of 7- 9.5 due to chemical reactions.

Burdock (2011) studied the effect of pH on triphenyl formazan yield at varying level of temperature and time. The result showed that pH above 7 yielded low amounts of TF, whereas the pH of 6 yielded more TF at all incubation times and temperatures.

3.8.3. *Temperature*

The rate of Triphenyl tetrazolium chloride reduction takes place with increasing

temperature (Mahmoud and Ghaly, 2006; Trevors, 1984). Trevors (1984) showed that increases in temperature up to 50 ºC resulted in a higher yield of triphenyl formazan by soil microorganism. By increasing the temperature, the rate of TTC reduction to coloured formazan was greater than at lower temperatures.

Burdock et al. (2011) stated that increasing the temperature by 10° C above optimum temperature exhibited negative effect on the dehydrogenase activity. This may have been due to enzyme inhibition at higher temperature levels. For all incubation temperatures examined (22°C, 30°C,40°C and 50°C), the highest TF yield was obtained at temperature of 22°C and 30°C than those observed at higher tmepratures (40°C and 50°C).

Breed (1957) reported that *S.venezuelae* reached optimal growth with the lower incubation temperature range of 28°C-30°C. Doull et al. (1993) reported a reduction in the growth of *S.venezuelae* at higher temperatures of 37°C - 42°C, compared to that at the control temperature of 27°C. Therefore, these studies support a temperature of 30°C as an optimum temperature for measuring the dehydrogenase activity of *S.venezuelae* using the TTC test.

3.8.4. *Incubation Time*

Burdock et al. (2011) state that the optimal time for TF yields was greater when the incubation time period for *Streptomyces venezuelae* was longer. They studied incubation time periods between 1 and 4 hours and found that TF yield was greater at the higher incubation period of 4 hours. Brooks et al. (2012) used a lesser incubation time (1 hour) period during shocking of *Streptomyces venezuelae* for secondary metabolite production and stated that shorter time is more practical to use for measuring cell growth and activity.

Mahmoud and Ghaly (2006) reported that TF yield from *A.niger* increased gradually with incubation time from 1.5 to 4.5 hours. Ghaly and Ben-Hassan (1993) reported similar effects of increasing incubation time for both *Candida pseudotropicalis* and *Kluyveromyces fragilis* grown in cheese whey.

Ross (1971) reported a low TF yield after an incubation time of 1 hour. They obtained similar results with shorter incubation times (2 and 4 hours) using 5-Cyano-2, 3-ditolyl tetrazolium chloride (CTC) salt. They concluded that TF yield increased exponentially with time greater than 4 hours.

3.8.5. *TTC Concentration*

The concentration of TTC should be low enough to avoid inhibition of bacterial growth, but high enough to allow color formation. More concentration of TTC is toxic and causes poor colour development by destroying cells. The TTC concentration used for dehydrogenase analysis depends on the type of bacteria. (Hurwitz and Carthy, 1986; Mustakallio and Ahos, 1955; Senyk et al., 1987).

Ghaly and Mahmoud (2006) reported that an increase in TTC concentration (from 5 to 15 g/L) indicated higher TF yield by 28% in *Asperigllus niger*. The authors state that in some case, the salt may act as an inhibitor by binding to active sites of the enzyme thereby make the enzyme-substrate complex unreactive.

Tengerdy et al. (1967) and Ohara and Saito (1995) indicate that tetrazolium salt in higher concentration may inhibit the growth of microorganism to some extent, while Shuler and Kargi (1992) state that higher concentration of tetrazolium salt in the medium results in the inactivation of bacterial cells or cell death, causing an inhibitory effect on enzymatic activity.

Altman (1969) reported that intact microbial cells and subcellular membranes can combine to form barriers for the entry of tetrazolium salts, where low concentration of TTC salt at the enzymatic reaction site may be a rate limiting factor even if the concentration in the medium is sufficient.

Altman (1976) reported that MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium-bromide) (414 g/mol) is able to penetrate mitochondrial membrane more effectively than NT (3,3'-(4,4'-biphenylylene)-bis-(2,5-diphenyl-2H-tetrazolium chloride) because it has low molecular weight (668 g/mol).

Bernas and Dobrucki (2000) suggested that MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) can quickly penetrate intact cell membranes because of its low molecular weight, while CTC (5-cyano-2,3-ditoyl tetrazolium chloride) remains extracellualr and require membrane permeable electron carrier to be reduced effectively. Based on these studies, it suggest that TTC will be more likely to penetrate the cell membranes as it has a smaller molecular weight (334.8 g/mol) in comparison to the other comman tetrazolium salts (Table 3.8).

Altman (1969) and Altman (1976) rated TTC having the lowest reducibility when compared with other tetrazolium salts and found their activity affected by the presence of O_2. The study indicated that one electron reduction of TTC produces a TTC free radical which could further reduced by the increase in another electron to TF. In the presence of O_2, the TTC radical reduces O_2 to superoxide and itself becomes oxidised back to TTC.

3.9. *Factors Affecting the Growth of Streptomyces venezuelae and Jadomycin Production*

There are several factors that affect the growth of *Streptomyces venezuelae* and the production of jadomycin. These include temperature, pH and nitrogen concentration in the media.

3.9.1. *Temperature*

Temperature is a vital environmental factor for bacterial growth and product fermentation (Zheng et al., 2001). Each organism shows minimum, maximum and optimum temperatures for the growth. These are known as cardinal temperature and are characteristic of an organism, although they depend upon other environmental factors such as the pH and nutrient availability (Adams and Moss, 1995). The rate of growth decreases precipitously above the optimal growth temperature while it decreases more gradually below the optimum (Doyle et al., 1997). Microorganisms can be differentiated into several physiological groups based on their temperature requirements (Adams and Moss, 1995). These classified groups are thermophiles (45°C to 65°C), mesophiles (15°C to 40°C) and psychrotrophs (-10°C to 25°C).

The temperature exhibits a significant influence over the physiological and chemical activity of the bacteria. Every species has a standard temperature for growth and production of bioactive metabolites (Shuler and Kargi, 1992). Though bacteria can tolerate extreme temperature, the optimum range for their survival and activity is rather narrow. Most of the soil bacteria (in this case *Streptomyces venezuelae*) are mesophillic (25-40°C).The temperature range over which the high titers of secondary metabolites are produced is normally narrower than the temperature range in which the producing microbes grow or primary metabolism occur. Growth of the microorganism at the sub-optimal temperature causes reduction in demand for the substrate supporting primary metabolism. The growth associated metabolism is more sensitive to thermal downshifts than the secondary metabolism (Walsh, 2003; Shapiro, 1989).

Growth Temperature: Most of the *Streptomyces* are mesophilic organisms requiring moderate temperature conditions. The growth range is between 15 and 40˚C with optimum being 32 ˚C for actinomycetes. Wang and Vining (2003), Jakeman et al. (2004) and Burdock et al. (2011) reported that *Streptomyces venezuelae* ISP5230 have a temperature growth range of 15-40°C, with an optimum growth temperature of 30°±2°C.

Production Temperature: The production of secondary metabolite can be triggered by heat shock at a temperature higher than the maximum growth temperature. Kavitha and Vijayalakshmi (2009) and Singh (1992) studied the effect of temperature on the antibiotic yield of *Streptomyces venezuelae* and found that at 42°C, *Streptomyces venezuelae* expressed heat shock genes that initiated the sporulation which favored antibiotic production. Doull et al. (1994) reported that the secondary metabolite production was absent when the bacteria was maintained at 27˚C and jadomycin started to accumulate when the bacteria was subjected to 42˚C.

3.9.2. *pH*

The hydrogen ion concentration (pH) in the fermentation media affects the cellular enzymes

of bacteria (Shuler and Kargi, 1992). The soil environment has a definite influence over *Streptomyces venezuelae*. Other soil organisms like blue-green algae or protozoa favors neutral or alkaline environments with a pH ranging between 4.5 and 8.0. Fungi prefer acidic environment with pH ranging between 4.5 and 6.5. Actinomycetes such as *Streptomyces venezuelae* prefer slightly alkaline conditions that favor both growth and secondary metabolite production (Jakeman et al., 2006; Doull et al., 1994).

Many of the biological and chemical systems are influenced by the pH. The pH can be controlled either by adding an acid or a base to keep it constant during the fermentation process (Ben-Hassan et al., 1991). Most *Streptomyce*s have a pH optimal for growth ranging between 6 and 8 and beyond this level the rate of growth rapidly diminishes. The pH of the medium used not only affects the growth but also affects the mycelial morphology and secondary metabolism. To obtain the desired product, the culture is said to be maintained at the optimum pH within ¬± 0.2 units (Shapiro, 1989). Inoue et al. (1982) observed that the optimal pH requirement for streptomycin production from *S. griseus* was 6.5 and at a pH of 7, little or no neomycin production was observed (Sebek, 1955).

3.9.2.1. pH for Growth

The pH of the media greatly affects the growth of bacteria and the cellular activities. Jakeman et al. (2004) reported that maintaining the pH between 6 and 7.8 contributed to a higher rate of growth and antibiotic production. Jakeman et al. (2006) studied the growth of the *Streptomyces venezuelae* and found that a pH range of 5.5 - 11.5 was effective. Poor growth of *S.venezuelae* was observed at a pH between 4 and 6, and a pH of 7 resulted in maximum growth. Kontro et al. (2005) reported that *Streptomyces* failed to grow in an extreme acidic environment, while in a neutral pH environment of 7 they actively increased in number and showed maximum growth rate. To buffer fermentation media to neutral pH, MOPS (3-(N-morpholino) propanesulfonic acid) has been used for *Streptomyces* (Kendrick et al., 1982).

3.9.2.2. pH for production

Production of various antibiotics is affected by the pH (above or below 7). Kavitha and

Vijayalakshmi (2009) reported high antibiotic yield at a pH of 6.5 – 7 for *Streptomyces tendae* TK-VL_333. In the study by Glaze brook et al (1990), MOPS was used to maintain the pH of the media for every carbon source except glucose in lactose-sulfite broth (LS) media. MOPS has also been used in the production media to maintain the pH for active metabolite production (Jakeman et al., 2006; Burdock et al., 2008). Jakeman et al. (2006) reported that the yield of jadomycin titers was lower at higher concentrations of MOPS whereas at lower MOPS concentrations the yield was greater. When the culture was subjected to lower MOPS (10mM), the culture pH dropped one unit over the first 24 hours, but was maintained for next 48 hours.

3.9.3. *Nitrogen*

Nitrogen constitutes about 10% of the dry mass of the bacteria. It is prominently found in the nucleic acids and proteins, where it accounts for 75% of the dry weight in the growing cells (Stouthamer, 1973). The factors influencing the nitrogen metabolism can deeply affect the physiological status of a bacterium by a severe response to amino acid starvation (Switzer et al., 1985; Gallant, 1979). Nitrogen as a nutrient (in metabolism) has greater influences during the growth phases, trophophase and the final yield of secondary metabolite (antibiotic) achieved by the production cultures. Nitrogenous compound are involved in antibiotogenesis and affect the bacteria at the level of secondary metabolism either through their modulation of biosynthesis or their availability as substrates for the antibiotic synthetase (Shapiro, 1989).

Ammonia present in the media is assimilated to amino acids via amino transferases and glutamate (Lightfoot et al., 1988). In the chloramphenicol production by *Streptomyces venezuelae*, glutamate dehydrogenase and glutamate synthase exhibited increased activity in the cultures taking in ammonium nitrogen and reduced activity with the depletion of ammonium or poor nitrogen sources. Shapiro (1989) studied that the antibiotic biosynthesis is correlated and controlled by activity that regulates the assimilation of nitrogen, glutamate dehydrogenase and glutamate synthase are the most favourable enzymes that prove the regulatory linkage.

Shapiro et al. (1984) and Shapiro and Vining (1983) reported that when *S.venezuelae* was cultivated in the medium with the ammonium nitrogen source, the nitrogen pool of low molecular weight was comprised of glutamate and glutamine. By exposing the culture to anaerobic condition, the growth ceased abruptly and the glutamine/glutamate pool rapidly decreased and resulted in the accumulation of large amount of alanine. The simultaneous uptake of the ammonium and nitrate by *Streptomyces venezuelae* did not occur, however this organism simultaneously used amino acids and nitrates when these two nitrogen sources were supplied in equimolar amount. Shapiro and Vining (1984) reported that the utilization of nitrates by *Streptomyces venezuelae* progressed faster and more rapidly with the amino acids than in their absence.

Ying et al. (2006) cultured the strain of *Streptomyces venezuelae* GY1 in different nitrogen sources (mass concentration of the nitrogen was $0.1 \ gL^{-1}$) and found that organic nitrogen was better than inorganic nitrogen for cell growth and $NaNO_3$ was considered the best sole nitrogen source for enzyme production. *Streptomyces* can intensively use amino acids either as carbon or nitrogen source. The ability of the microbe to use the amino acid as a source of nitrogen depends upon the ability to move the amino acid into the cell, discase it of its nitrogen and incorporate that nitrogen into the metabolic scheme (Shapiro, 1989).

Gottlieb and Ciferri (1956) reported that *Streptomyces venezuelae* were able to deaminate many amino acids. The amino acids readily deaminated by the resting suspension of the microbe also resulted in good growth in the media containing the amino acid as the sole nitrogen source.

Gottlieb and Legator (1953) reported that the *Streptomyces venezuelae* growth was conducted in the liquid culture in which the inorganic nitrogen was reserved during the organism's growth. It was observed that 86% of the nitrate nitrogen disappeared within the first 12 hours and 93 % disappeared within 48 hours. The nitrogen drop in the early stage reflects the nitrate removal while the increase level indicates lysis caused by mycelium. So, by the increase in the nitrogen concentration the growth and the total weight of the *Streptomyces venezuelae* had begun to cease or decrease.

Dupuis et al. (2011) and Jakeman et al. (2005) reported that *Streptomyces venezuelae* ISP5230 cultures were grown in production media using the amino acids as their sole nitrogen source. After the cultures had grown, they were subjected to the ethanol shock. Soon after the ethanol shock, they were left for 24 hours before the cells were harvested and jadomycin extracted. The delay in the colour build up was due to difficulty in metabolizing the amino acid analogues.

The electrospray ionisation mass spectroscopy extracts obtained from the *Streptomyces venezuelae* ISP 5230 cultures that were grown with non–natural amino acids (L-amino acids) and 20 different naturally occurring amino acids (D-amino acids) were studied by Jakeman et al (2005). It was concluded that non-natural amino acids could be incorporated into jadomycin analogues by *Streptomyces venezuelae* ISP5230 in addition to the natural L-amino acids.

Different nitrogen sources have been used by Doull et al (1994) to derive the active yield of secondary bioactive compounds from the strains as shown in Table 3.9. The suitable condition for the production of jadomycin by the *Streptomyces venezuelae* ISP5230 was analyzed. It was observed that the nature of the nitrogen was found to be important. Different jadomycin analogues were formed when the other amino acids were replaced with the L-isoleucine as the nitrogen source in the medium. Higher jadomycin B yields were obtained with higher L-isoleucine and D-galactose concentration in the production medium.

Table 3.9. Effect of varying l-amino acid nitrogen on the jadomycin production from cultures of *Streptomyces venezuelae* supplemented with ethanol (6%v/v) (Doull et al., 1994).

		Color of Culture Medium		
Black	Orange	Purple	Red	Yellow
Tryptophan	Alanine	Threonine	Glycine	Arginine
	Glutamic acid		Lysine	Asparagine
	Isoleucine		Phenylalanine	Aspartic acid
	Leucine		Tryosine	Cysteine
	Methionine			Glutamine
	Serine			Histidine
	Valine			

CHAPTER 4. MATERIALS AND METHODS

4.1. *Experimental Materials*

4.1.1. *Glassware*

The glassware used in this research included test tubes, beakers, reagent bottles, Pyrex bottles and pipettes. All the glassware were washed using soap and hot water, rinsed with distilled water and autoclaved before use in the experiments.

4.1.2. *Chemicals*

Tris(hydroxymethyl)aminomethane, 2,3,5-triphenyl formazan (TF), and 2,3,5-triphenyl tetrazolium chloride (TTC) were obtained from Sigma-Aldrich (Oakville, Ontario, Canada) and used to measure the dehydrogenase activity during environmental shock. Anhydrous ethanol and methanol were obtained from Tupper Building Stores at Dalhousie University (Halifax, Nova Scotia, Canada). Ethanol was used to shock *S.venezuelae* for jadomycin production. Methanol was used to shock *Streptomyces venezuelae* for jadomycin production and as an extraction solvent for removing TF from within the cells during dehydrogenase measurement tests. The apramycin sulfate salt used during MYM agar plate preparation was obtained from Sigma-Aldrich (Oakville, Ontario, Canada). All the reagents and media components were obtained from Bio-Shop (Burlington, Ontario, Canada).

4.1.3. *Equipment*

The equipment used in the experiments were: Hund H500 Series Phase Contrast Microscope (Model Number H500, Helmut Hund GmbH, Wetzlar Germany), Series 25 Incubator Shaker (New Brunswick Scientific Co. Inc, Edison, New Jersey, USA), a Precision 2870 Series Water Bath Shaker (Model Number 2870, Thermo Scientific, Marietta, Ohio, USA), a Precision 280 Series Water Bath (Thermo Scientific, Ohio, USA), a Large Stand up Incubator (Model Number 2020, VWR International, Cornelius, Oregan, USA), Isotemp 655 F oven (Fisher Scientific, Marietta, Ohio, USA), Sorvall RT1 Centrifuge (Thermo Scientific, Marietta, Ohio, USA), Branson R-series Sonicator (Model Number 2510, DTH, Danbury,

Connecticut, USA), Metler AE 200-S digital weigh balance and PM 4600 balance (Mettler-Toledo International Inc., Mississauga, Canada), Orion 5 Star pH meter (Model Number B34326,Thermo Scientific, Lowell, Massachusetts, USA) and Ultra Basic Denver pH Meter (Model Number UB-10, Denver Instrument,Bohemia, New York USA), Genesys 10 S UV-VIS spectrophometer (Thermo Scientific, Marietta, Ohio, USA) and Barnstead Thermolyne Maxi Mix (Model Number 16700, Thermolyne Corporation, Hampton, New Hampshire, USA).

4.1.4. *Bacterial Culture*

A starter plate of *Streptomyces venezuelae* ISP5230 was obtained approximately 3 weeks after streaking from the Jakeman Laboratory, College of Pharmacy, Dalhousie University (Halifax, Nova Scotia, Canada).

4.2. *Experimental Design*

The experimental work was divided into six parts as shown in Table 4.1.

In the first set of the experiments, the growth of *S.venezuelae* (scraped from the starter plate) in nutrient rich MYM growth media was monitored for 65 hours.

In the second set of the experiments, the vegetative *S.venezuelae* cells were collected at different time periods (18, 21 and 24 hour), placed in nutrient deprived amino acid rich MSM production media and shocked at the time of inoculation as stated by Jakeman et al. (2006) and Dupius et al. (2010).

In the third set of the experiments, the effects of heat shock treatment (35, 40, 45 and 50°C) and exposure time (0.5 and 1 h) were investigated. At the end of exposure to the heat treatment, the temperature was brought back to the optimum growth temperature of 30°C.

The fourth set of the experiments was designed to determine the effect of alcohol shocking using four ethanol or methanol concentrations (3, 4.5, 6 and 7.5%). The shocking was carried out immediately after the bacteria were transferred to the production media.

The fifth set of the experiments was devoted to the study of multiple shocking at various

Table 4.1. Experimental plan for the optimization of various shocking method.

Experiment	Factors	Parameters	No. of Replicates	Description
Growth	Time	65 h	3	Growth of *S.venezuelae* in nutrient rich MYM media
Growth Transfer Stage	Time	18, 21 and 24 h	3	*S.venezuelae* grown for 18, 21 and 24 hour in MYM media, transferred to production media and shocked.
Heat shock	Temperature	35, 40, 45 and 50 °C	3	*S.venezuelae* heat shocked at various temperatures and brought back to 30 °C.
	Exposure Time	0.5 and 1 h		
Alcohol Shock	Concentration	3, 4.5. 6 and 7.5% v/v	3	*S.venezuelae* ethanol and methanol shocked at the time of inoculation.
	Alcohol Type	Ethanol and Methanol		
Multiple Alcohol Shock	Ethanol	3 % v/v	3	*S.venezuelae* ethanol shocked at multiple times.
	Time of Shock	(0 h), (0 and 3 h) and (0,3 and 6 h)		
Nutrient and Alcohol Shock	Nitrogen Conc.	45, 60 and 75mM	3	Different ethanol concentration was used to shock *S.venezuelae* while varying nitrogen concentration at the time of inoculation.
	Ethanol	3, 4.5 and 6% v/v		

times (0 h, 0 and 3 h and 0, 3 and 6 h using 3% v/v ethanol) following the transfer of bacteria to production media.

The sixth set of the experiments was designed to the study of effect of shocking with various ethanol concentrations (3, 4.5 and 6 %) while varying nitrogen concentrations (45, 60 and 75 mM) in the MSM production media at the time of inoculation.

4.3. *Preparation of Media, Inoculum and Dehydrogenase Reagents*

4.3.1. *Preparation of Bacterial Growth Medium*

Preparation of Maltose-yeast extract-malt extract (MYM) agar and broth was carried out according to the method described by Jakeman et al. (2006). The MYM agar was prepared by mixing 4 g maltose, 4 g yeast extract, 10 g malt extract, 1.9 g 3-(N-morpholino) propanesulfonic acid MOPS buffer and 15 g agar in 1L of distilled water using a magnetic stirrer (Model 310T. Fisher Scientific, Ottawa, Canada). A similar procedure was followed for the preparation of MYM broth without the addition of agar. The components of both MYM agar and broth are shown in Tables 4.2 and 4.3.

125 mL of the prepared growth media was distributed to 250 mL conical flasks. The flasks were sealed with foam inserts, covered with aluminium foil and autoclaved (Sterile max, Harvey/Barnstead International of thermo Fisher Scientific, Ottawa, Ontario Canada) on the liquid setting (121ºC, 20 Pa) for 15 minutes. Autoclaved flasks were left to cool at room temperature before use.

4.3.2. *Preparation of Jadomycin Production Medium*

The buffered mineral salt solution (MSM production media) contained glucose as carbon source, isoleucine as nitrogen source, trace minerals, salt, ferrous and phosphate compounds. Initially, a stock solution of production media was used for the production process and was prepared using the components shown in Table 4.4.

The salt stock solution was prepared by placing 5 g NaCl (sodium chloride) and 5 g CaCl$_2$ (calcium chloride) into a 500 mL volumetric flask and adding distilled water to

Table 4.2. MYM broth components.

Component	Concentration (g/L)
Maltose	4.0
Yeast Extract	4.0
Malt Extract	10.0
MOPS	1.9

*Source - Bio-Shop (Burlington, Ontario, Canada)

Table 4.3. MYM agar components.

Component	Concentration (g/L)
Maltose	4.0
Yeast Extract	4.0
Malt Extract	10.0
MOPS	1.9
Agar	15.0

* Source - Bio-Shop (Burlington, Ontario, Canada)

Table 4.4. MSM Production media components

Component	Quantity(per L distilled water)	Chemical Formula
Magnesium Sulfate	0.4 g	$MgSO_4$
MOPS	1.9 g	$C_7H_{15}NO_4S$
Salt Solution	9.0 mL	
Sodium Chloride	5.0 g	$NaCl$
Calcium Chloride	5.0 g	$CaCl_2$
0.2% Ferrous Sulfate Solution	4.5 mL	$FeSO_4 \cdot 7H_2 0$
Trace Mineral Solution	4.5 mL	
Zinc Sulfate	880 mg	$ZnSO_4 \cdot 7H_2 0$
Cupric Sulfate	39 mg	$CuSO_4 \cdot 5H_2 0$
Manganese Sulfate	6.1 mg	$MnSO_4 \cdot 4H_2 0$
Boric Acid	5.7 mg	H_3BO_3
Ammonium Molybdate	3.7 mg	$(NH_4)_6Mo_7O_{24} \cdot 4H_2 0$
Isoleucine	7.8 g	$C_6H_{13}NO_2$
Glucose	20.0 mL	$C_6H_{12}O_6$
Phosphate Solution	5.5 mL	
Sodium Phosphate Monobasic	1.24g	$NaH_2PO_4 \cdot H_2O$

bring the total volume to 500 mL. The solution was thoroughly mixed on a magnetic stirrer (Model No: 120S, Fisher Scientific, Ottawa, Ontario, Canada).

The 0.2 % ferrous sulphate stock solution was prepared by placing 1 g $FeSO_4 \cdot 7H_2O$ in a 500 mL volumetric flask and adding distilled water to bring the total volume to 500 mL. The solution was thoroughly mixed on a magnetic stirrer (Model No: 120S, Fisher Scientific, Ottawa, Ontario, Canada).

The trace mineral stock solution was prepared by placing 880 mg $ZnSO_4 \cdot 7H_2O$ (zinc sulfate), 39 mg $CuSO_4 \cdot 5H_2O$ (cupric sulfate), 6.1 mg $MnSO_4 \cdot 4H_2O$ (manganese sulfate), 5.7 mg H_3BO_3 (boric acid) and 3.7 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (ammonium molybdate) in a 1 L volumetric flask and adding distilled water to bring the total volume to 1 L. The solution was thoroughly mixed on a magnetic stirrer (Model No: 120S, Fisher Scientific, Ottawa, Ontario, Canada).

The glucose stock solution was prepared by transferring 5.94 g glucose to a 20 mL volumetric flask and adding distilled water to bring the total volume to 20 mL. The solution was thoroughly mixed on a magnetic stirrer (Model No: 120S, Fisher Scientific, Ottawa, Ontario, Canada).

The phosphate stock solution was prepared by placing 1.24 g $NaH_2PO_4 \cdot H_2O$ (sodium phosphate monobasic) in 1 L volumetric flask and adding distilled water to bring the total volume to 1 L.

The production medium was prepared by transferring 0.4 g $MgSO_4$ (magnesium sulfate), 1.9 g MOPS, 7.8 g isoleucine, 9 mL salt solution, 4.5 mL 0.2 % $FeSO_4 \cdot 7H_2O$ (ferrous sulfate) and 4.5 mL trace mineral solution to a 1 L volumetric flask and adding distilled water to bring the volume to 1L. The prepared solution was autoclaved (Sterile Max, Harvey/Barnstead International of Thermo Fisher Scientific, Ottawa, Ontario, Canada) on the liquid setting (121ºC, 20 Pa) for 15 minutes. 20 ml of glucose and 5.5 ml phosphate were placed in a separate flask and added to the production medium at room temperature after autoclaving to avoid precipitation. Several 1 L solutions were prepared and stored. The final solutions were distributed evenly in 250 mL sterilized flasks and stored at 4 ºC in refrigerator until needed.

4.3.3. *Preparation of Maltose-yeast extract-malt extract (MYM) agar Plates*

Four 125 mL autoclaved agar flasks were used to fill 25 average sized (100mm dia. x 15mm H) petri plates. Using a laminar flow hood (Model No. FFV24A, Microzone Corporation, Ontario, Canada) to provide a sterile environment, 25 mg apramycin sulphate salt (aminocyclitol antibiotic) (Sigma-Aldrich, Oakville, Ontario, Canada) was added to the 500 mL agar medium and the medium was then gently poured into each petri dish to about 3 mm depth. The agar was allowed to solidify in the petri dishes by leaving them on a flat sterile surface for 10 minutes. Each plate was wrapped tightly using Parafilm and refrigerated at 4°C until used for streaking and serial dilution procedure.

4.3.4. *Preparation of Inoculum*

Ten stock (MYM) agar plates of *Streptomyces venezuelae* were prepared from the original starter plate and used as an inoculum for the growth and shocking experiments. To prepare the bacteria stock plates, several colonies were separated aseptically and transferred to a 250 mL erlenmeyer flask containing 125 mL MYM growth media (pH 7). The neck of the flask was sealed with a cotton plug and aluminum foil and incubated in an environmentally controlled shaker (Series 25, New Brunswick Scientific Co. Inc., New Brunswick, New Jersey, U.S.) at 30°C , 250 rpm for 20 hours. After 20 hours, samples were viewed through a phase contrast microscope (HundWetzlar H500, Wetzlar, Germany) for structure verification. The elongated and branched structure appeared as shown in Figure 4.1. A disposable loop was used to streak the prepared MYM agar plates with growth culture and incubated at 30°C for 14 days until visible colonies were formed. The colonies appeared as a bright, crusty layer indicating the presence of spores (Figure 4.2). The experiments were started with spores in order to minimize the errors in inoculum size.

4.3.5. *Preparation of Dehydrogenase Activity Reagents*

The dehydrogenase activity regents were prepared as shown in Table 4.5. The 2,3,5-triphenyl tetrazolium chloride (TTC) - glucose solution was prepared by combining 1 glucose and 2 g TTC in a 100 mL volumetric flask. The total volume was brought to 100 mLwith distilled water. The prepared TTC-glucose solution was stored in the dark at 4°C.The Tris (hydroxymethyl) aminomethane (Tris) solution was prepared by adding 6 g Tris buffer with
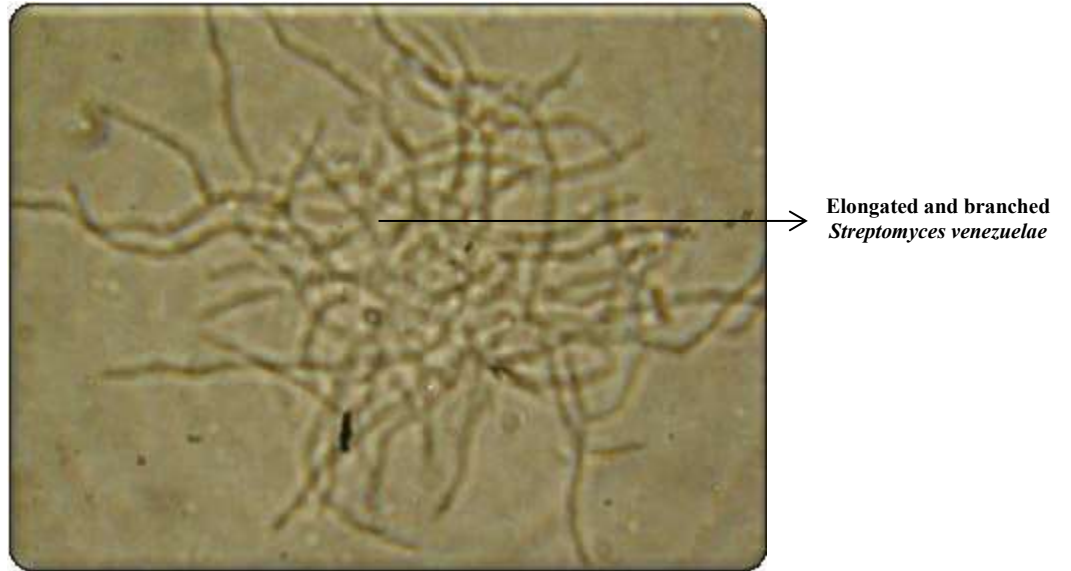
Figure 4.1. Elongated and branched *Streptomyces venezuelae* mycelium after 20 h in MYM growth media (100 x magnifications).
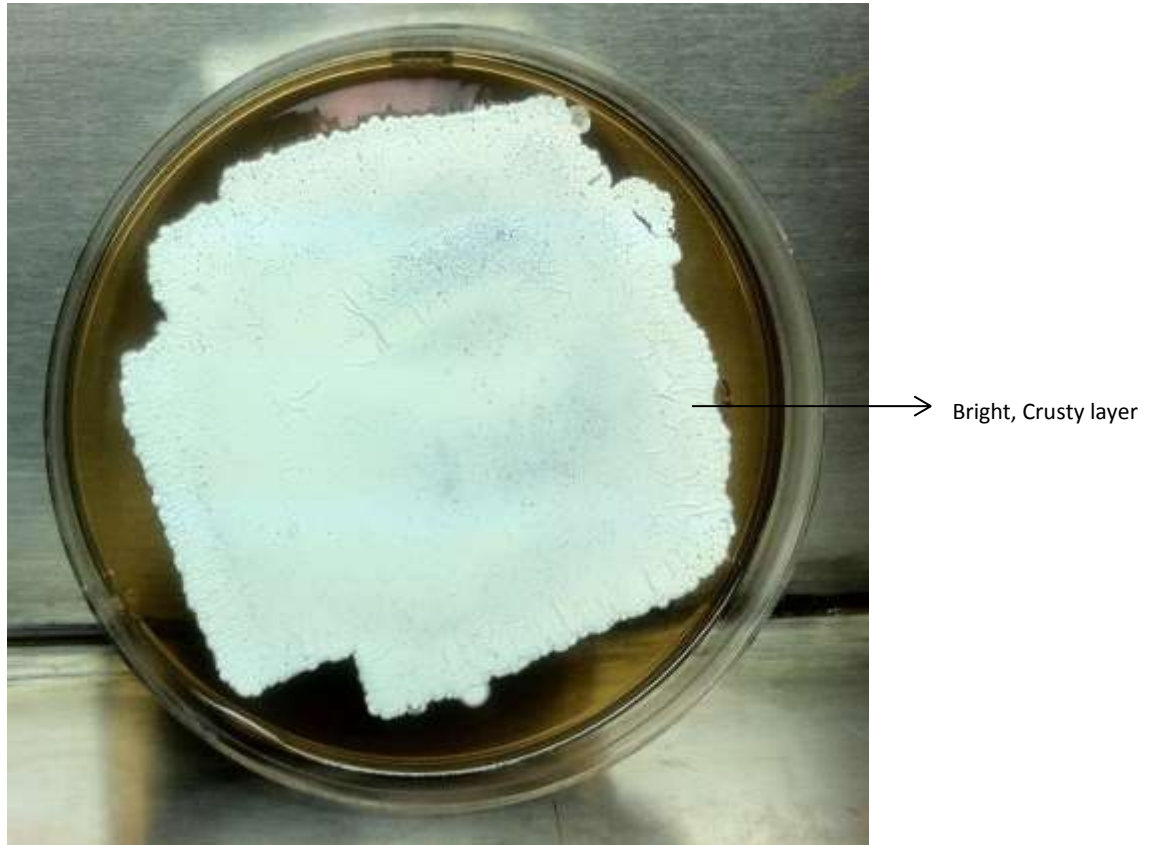
Figure 4.2. *Streptomyces venezuelae* ISP5230 spore plate used for all experiments after incubating at 30 ºC for 14 d.

Table 4.5. Reagent for dehydrogenase activity tests.

Component	Quantity (g/mL)
2,3,5-triphenyl tetrazolium chloride (TTC)	2g /100mL
Glucose	1g /100mL
Tris(hydroxymethyl)aminomethane (Tris) solution	6g /1000 mL

20 mL 1 N NaOH to a 1 L beaker. The total volume was brought to 1 L with distilled water and stored at room temperature.

4.4. *Experimental Procedure*

4.4.1. *Growth Experimnt*

This experiment was devoted to the study of *S.venezuelae* growth. Three 250 mL conical shake flasks were each filled with 150 mL of MYM broth, plugged with foam inserts and then covered with aluminium foil. They were autoclaved (SterileMax, Harvey/Barnstead International of Thermo Fisher Scientific, Ottawa, Ontario, Canada) at the liquid setting (121 ºC and 20 Pa) for 15 minutes. After cooling to room temperature, the MYM broth in each flask was inoculated with disposable loop of *S. venezuelae* ISP5230 scraped from 14 day old MYM agar plates. The flasks were then incubated in an environmentally controlled shaker (Series 25, New Brunswick Scientific Co. Inc., New Brunswick, New Jersey, U.S.) at 30 ºC and 250 rpm and were allowed to grow for 65 hours. Samples (8 mL) were taken from each flask at 4, 8, 16, 18, 20, 24, 30, 35, 40, 45, 50, 55, 60 and 65 h for analysis. Volumes of 4ml were used to measure absorbance at 600 nm, 2 mL were used for measuring dehydrogenase activity and 1 mL was used for image verification and plate count. The overall procedure is shown in Figure 4.3.

4.4.2. *Time of Culture Transfer Experiment*

This experiment was devoted to the determination of the appropriate time for transferring *S.venezuelae* culture from the growth medium to the production medium. Three growth periods (18, 21 and 24 h) after inoculation of growth medium were evaluated. The overall procedure is shown in Figure 4.4.

Nine 250 mL conical flasks were each filled with 150 mL of MYM broth, plugged with foam inserts and then covered with aluminium foil. They were autoclaved (SterileMax, Harvey/Barnstead International of Thermo Fisher Scientific, Ottawa, Ontario Canada) at the liquid setting (121 ºC and 20 Pa) for 15 minutes. After cooling to room temperature, the MYM broth in each flasks were incubated in environmentally controlled shaker (Series 25, New Brunswick Scientific Co. Inc., New Brunswick, New Jersey, U.S.) at 30 ºC and 250 rpm.
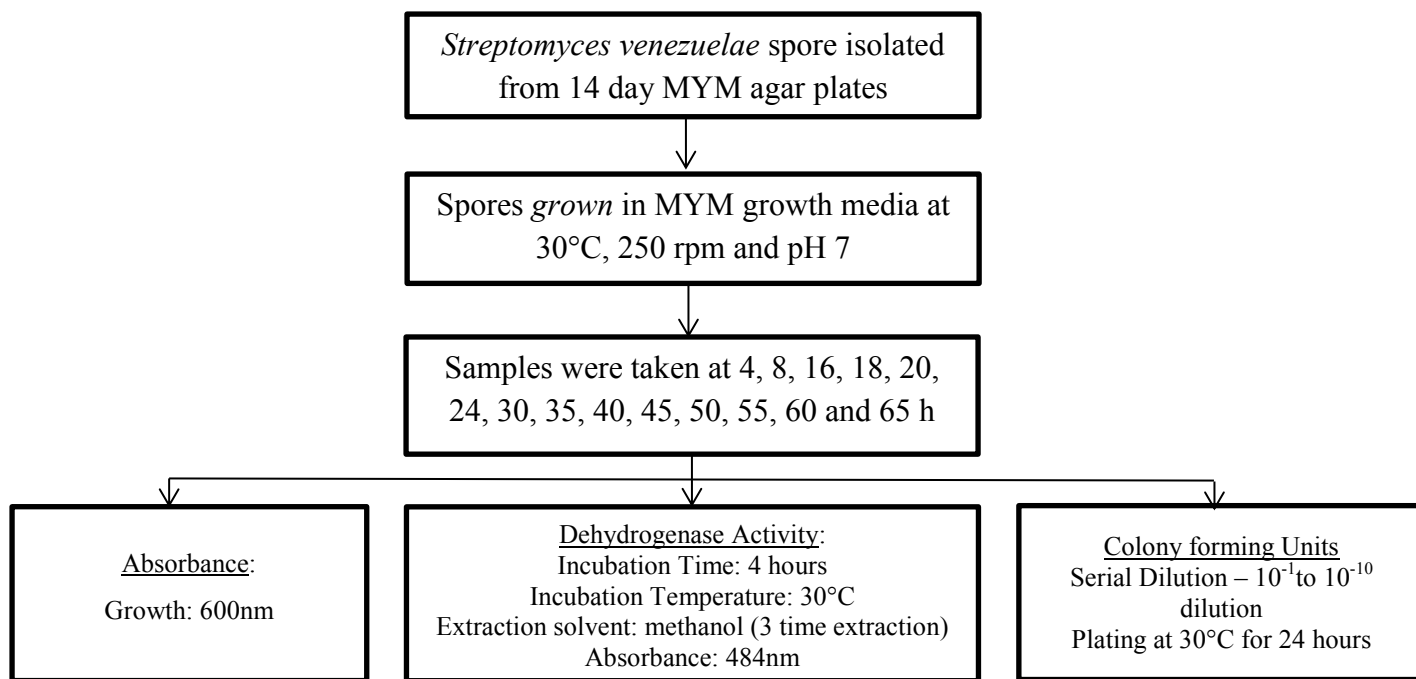
Figure 4.3. Growth of *Streptomyces venezuelae* ISP5230 VS1099 in nutrient rich MYM growth media.
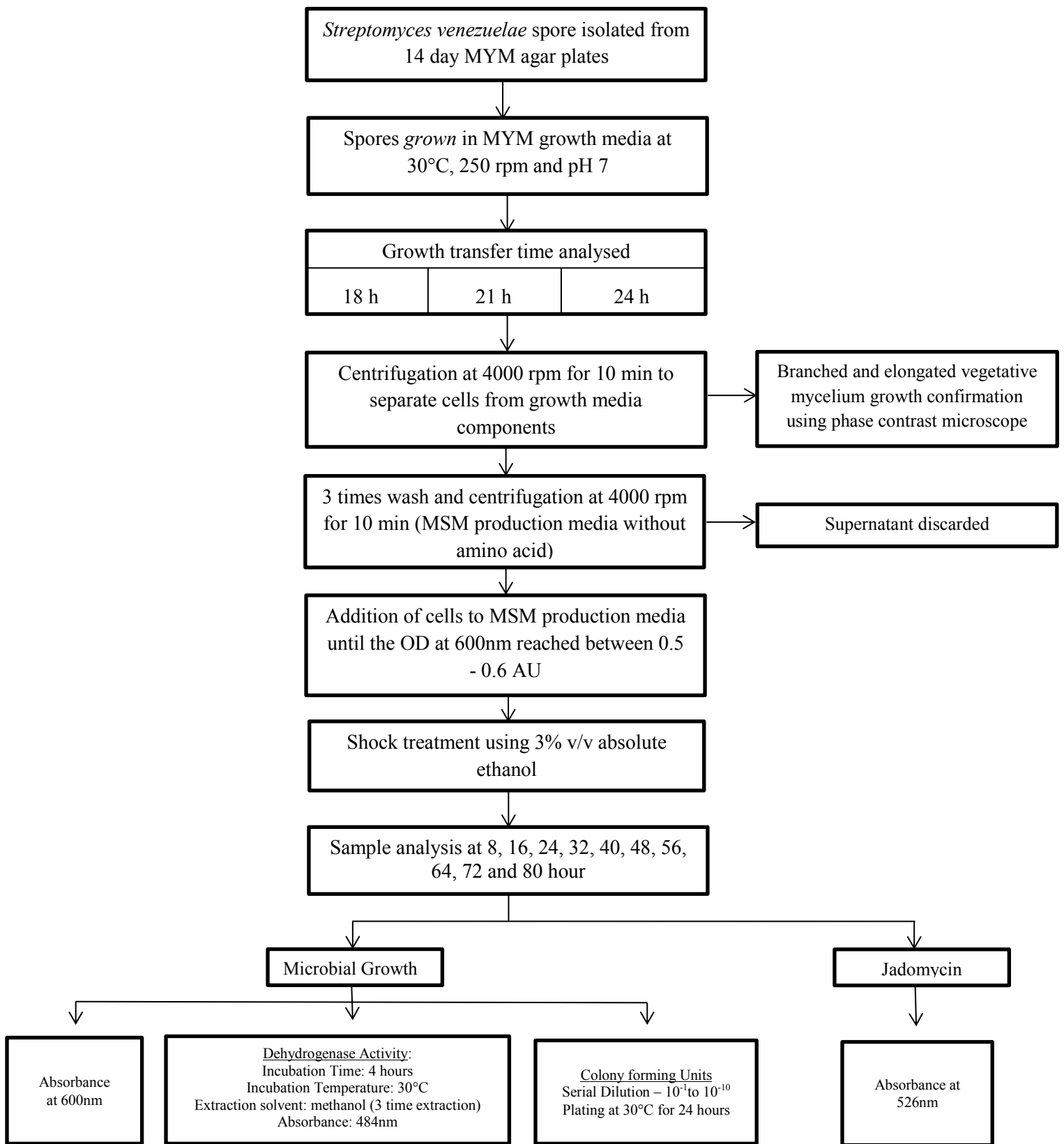
Figure 4.4. Procedure for the growth transfer experiment to shock in MSM media.

The *Streptomyces venezuelae* was allowed to grow in the culture media for 18 h in 3 flasks, 21 h in 3 flasks and 24 h in 3 flasks.

Nine samples were taken from each flask and placed in three 50mL centrifuge tubes. The tubes were centrifuged (Thermo Scientific, Ohio, USA) at 4000 rpm for 10 minutes. The supernatants were discarded and the cells obtained from each flask (3 tubes) were mixed together and marked as Trials 1, 2 and 3 for the 18 h, Trial 4, 5 and 6 for the 21 h and Trials 7, 8 and 9 for the 24h.

Three washings were carried out using MSM production media without amino acid (L-isoleucine) to remove the remaining growth media (MYM broth). For the first wash, 10 mL MSM production media without amino acid were added to a 50 mL centrifuge tube containing the clumped cells and vortexed for 3 minutes to disrupt the cells. Then, the sample were centrifuged (Thermo Scientific, Ohio, USA) at 4000 rpm for 10 minutes. The supernatant was discarded and the cells were collected. The same procedure was repeated 2 more times.

After washing three times with production media without amino acid, the cells were re-suspended in 20 mL production media with amino acid (pH 7-7.5). The suspended cell culture was used to inoculate nine 250 mL Erlenmeyer flask, each containing same 125 mL MSM production media (pH 7-7.5). 1 mL of the cell culture was transferred to each flask using 1 mL sterile pipette. The culture was allowed to grow until the OD 600nm reached between 0.5-0.6 AU as recommended by Jakeman et al. (2006). This was verified using spectrophotometer (Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada).

The cultures were shocked by adding 3% v/v anhydrous ethanol at 0 hour and left for 80 hour after shocking. The pH was adjusted to 7 using 1 N NaOH after 24 hours post shock and left unchanged for the remaining time of the 80 hours. Samples (11 mL) were taken from each flask at 8, 16, 24, 32, 40, 48, 56, 64, 72 and 80 h for microbial and jadomycin analyses. Volumes of 4 mL were used to measure the absorbance at 600 nm, 4 mL used to measure jadomycin absorbance at 526 nm, 2 mL used for measuring dehydrogenase activity and 1 mL was used for plate counting.

4.4.3. *Heat Shock Experiment*

Four *S.venezuelae* shocking temperatures (35, 40, 45 and 50°C) were evaluated in this experiment. The overall procedure is shown in Figure 4.5. Six 250 mL conical flasks were each filled with 150 mL of MYM broth and used for each temperature, 3 flasks for each exposure time (0.5 and 1 h). The flasks were plugged with foam inserts and then covered with aluminium foil. They were autoclaved (SterileMax, Harvey/Barnstead International of Thermo Fisher Scientific, Ottawa, Ontario, Canada) at the liquid setting (121 °C and 20 Pa) for 15 minutes. After cooling to room temperature, the MYM broth was inoculated with a disposable loop of *S. venezuelae* ISP5230 scraped from 14 day old MYM agar plates. The flasks were incubated in an environmentally controlled shaker (Series 25, New Brunswick Scientific Co. Inc., New Brunswick, New Jersey, U.S.) at 30 ºC and 250 rpm. The cultures were allowed to grow for 18 h in the MYM broth medium.

Samples were taken from each flask in 50 mL centrifuge tubes (Corning Centrifuge Tubes, Falcon) and centrifuged at 4000 rpm for 10 minutes. The supernatants were discarded and the cells were collected. Three times washing of *Streptomyces* cells was carried out using 10 mL MSM production media without amino acid. The cells were collected and used to inoculate six 250 mL erlenmeyer flaskscontaining 125 mL MSM production media (pH 7-7.5). The cultures were allowed to grow until the OD-600nm reaches between 0.5-0.6 AU as recommended by Jakeman et al. (2006). This was verified using spectrophotometer (Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada).

The flasks were then placed on the top of magnetic stirrer at 250 rpm (Thermolyne Maxi Mix, Thermolyne Corporation, Hampton, NH, USA) as shown in Figure 4.6 and kept inside an environmentally controlled large standing incubator (Model number 2020, VWR International, Cornelius, OR). The cultures were heated to the temperature of 35°C (three flasks for 0.5 h and three flasks for 1 h) and then transferred back to the environmentally controlled incubator shaker (25 Incubator Shaker, New Brunswick Scientific, Edison, New Jersey, USA) at 30°C and 250 rpm. Samples (11 mL) were taken from each flask at 8, 16, 24, 32, 40, 48, 56, 64, 72 and 80 h for microbial and jadomycin analyses, 4 mL were used to measure the absorbance at 600 nm, 4 mL were used to measure jadomycin absorbance at 526

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                    ┌─────────────────────────────────────────┐
                    │ Streptomyces venezuelae spore isolated    │
                    │ from14-21 day MYM agar plates             │
                    └─────────────────────────────────────────┘
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                    ┌─────────────────────────────────────────┐
                    │ Streptomyces venezuelae grown in MYM      │
                    │ broth at 30°C, 250 rpm, and pH 7 for 18 h │
                    └─────────────────────────────────────────┘
                                      │
                                      ▼
        ┌─────────────────────────────────────────┐         ┌──────────────────────┐
        │ Centrifuged at 4000 rpm for 10 min to     │────────▶│ Supernatant discarded │
        │ separate cells for washing                │         └──────────────────────┘
        └─────────────────────────────────────────┘
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        ┌─────────────────────────────────────────┐         ┌──────────────────────┐
        │ 3 washs and centrifugation at 4000 rpm    │────────▶│ Supernatant discarded │
        │ for 10 min (MSM production media without  │         └──────────────────────┘
        │ amino acid)                               │
        └─────────────────────────────────────────┘
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        │ Vegetative cells grown in MSM production  │
        │ media at 30°Cand 250 rpm till turbidity   │
        │ measurement at OD600nm reaches between     │
        │ 0.5-0.6 AU                                │
        └─────────────────────────────────────────┘
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Heat Shock: 35°C	Heat Shock: 40°C	Heat Shock: 45°C	Heat Shock: 50°C
Exposure: 0.5 and 1 h	Exposure: 0.5 and 1 h	Exposure: 0.5 and 1 h	Exposure: 0.5 and 1 h

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        ┌─────────────────────────────────────────┐
        │ Shifted back to 30°C, 250 rpm and pH      │
        │ adjusted after 24 hours post shock        │
        └─────────────────────────────────────────┘
                                      │
                                      ▼
        ┌─────────────────────────────────────────┐
        │ Samples taken at 8, 16, 24, 32, 48, 56,   │
        │ 64, 72 and 80 hours for analysis          │
        └─────────────────────────────────────────┘
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	Microbial Growth		Jadomycin
Absorbance at 600nm	Dehydrogenase Activity: Incubation Time: 4 hours Incubation Temperature: 30°C Extraction solvent: methanol (3 time extraction) Absorbance: 484nm	Colony forming Units Serial Dilution – 10^{-1} to 10^{-10} Plating at 30°C for 24 hours	Absorbance at 526nm

Figure 4.5. Procedure for the heat shock experiment.

Figure 4.6. Set-up for the heat shock experiment.

nm, 2 mL were used for measuring dehydrogenase activity and 1 mL was used for plate counting. The same procedure was followed with the temperatures of 40, 45 and 50°C.

4.4.4. *Alcohol Type and Concentration Experiemnt*

The effect of alcohol type and concentration on the yield of jadomycin were investigated. Two alcohols (ethanol and methanol) and four alcohol concentrations (3.0, 4.5, 6.0 and 7.5 % v/v) were evaluated. The overall procedure is shown in Figures 4.7. Three 250 mL conical flasks were filled with 150 mL of MYM broth. The flasks were plugged with foam inserts and then covered with aluminium foil. They were autoclaved (SterileMax, Harvey/Barnstead International of Thermo Fisher Scientific, Ottawa, Ontario, Canada) at the liquid setting (121 ºC, and 20 Pa) for 15 minutes. After cooling to room temperature, the MYM broth was inoculated with a disposable loop of *S. venezuelae* ISP5230 scraped from 14 day old MYM agar plates. The flasks were incubated in environmentally controlled shaker (Series 25, New Brunswick Scientific Co. Inc., New Brunswick, New Jersey, U.S.A) at 30 ºC with 250 rpm. The cultures were allowed to grow for 18 hour.

Three Samples were taken from each flask in 50 mL centrifuge tubes (Corning Centrifuge Tubes, Falcon) and centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded and the cells were collected. Three washing of *Streptomyces* cells were carried outusing 10 mL MSM production media without amino acid as previously described. The cells were collected and used to inoculate six 250 mL flasks each containing 125 mL MSM production media (pH 7-7.5). The cultures were allowed to grow until the OD 600nm reached between 0.5-0.6 AU as recomended by Jakeman et al. (2006). This was verified using spectrophotometer (Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada).

Two types of anhydrous alcohol, (ethanol and methanol) with 3% v/v concentration were used to stress at 0 hour (3 flasks for each alcohol). After the addition of alcohol, the flasks were kept in an environmentally controlled incubator shaker (25 Incubator Shaker, New Brunswick Scientific, Edison, New Jersey, USA) at 30°C and 250 rpm for 80 hours. Samples (11 mL) were taken from each flask at 8, 16, 24, 32, 40, 48, 56, 64, 72 and 80 h for microbial and jadomycin analyses. 4 mL were used to measure the absorbance at 600 nm, 4 mL used to
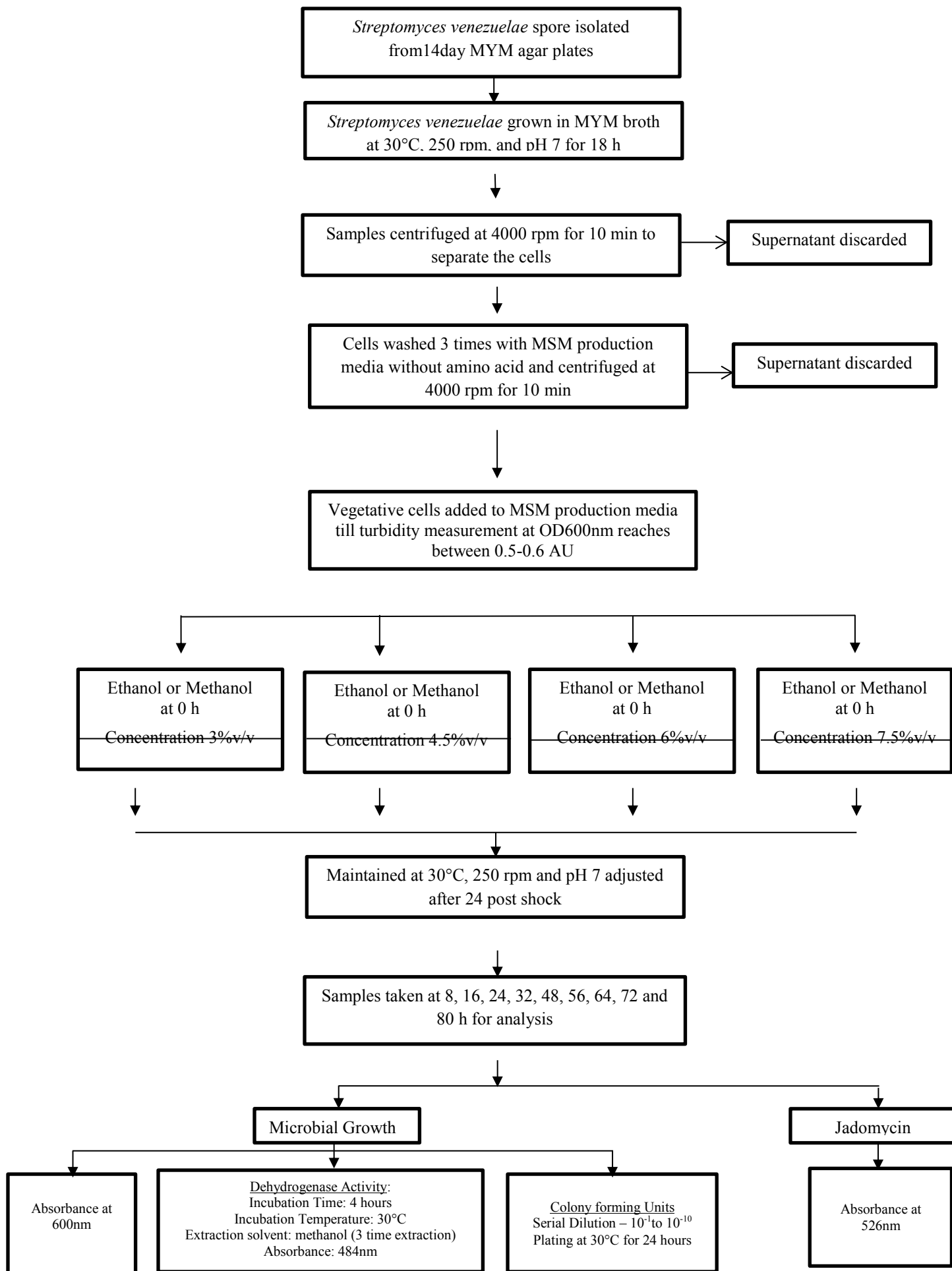
Figure 4.7. Procedure for alcohol type and concentration experiment.

measure jadomycin absorbance at 526 nm, 2 mL were used for measuring dehydrogenase activity and 1 mL was used for plate counting. The same procedure was followed with alcohol concentration 4.5, 6 and 7.5 % v/v for ethanol and methanol.

4.4.5. *Multiple Ethanol Shock Experiment*

This experiment was carried out to evaluate the effect of repeated shocking with 3% ethanol on the jadomycin yield. Several shocks were investigated (one shock at 0 h, two shocksat 0 and 3h and three shocks at 0, 3 and 6 h. The overall procedure is shown in Figure 4.8. Three 250 mL conical flasks were filled with 150 mL of MYM broth. The flasks were plugged with foam inserts and then covered with aluminium foil. They were autoclaved (SterileMax, Harvey/Barnstead International of Thermo Fisher Scientific, Ottawa, Ontario, Canada) at the liquid setting (121 ºC and 20 Pa) for 15 minutes. After cooling to room temperature, the MYM broth was inoculated with a disposable loop of *S. venezuelae* ISP5230 scraped from 14 day old MYM agar plates. The flasks were incubated in an environmentally controlled shaker (Series 25, New Brunswick Scientific Co. Inc., New Brunswick, New Jersey, U.S.) at 30 ºC and 250 rpm. The cultures were allowed to grow for 18 hours.

Three samples were taken from each flask and placed in 50 mL centrifuge tubes. The tubes were centrifuged at 4000 rpm for 10 minutes. The supernatants were discarded and the cells were collected. Three washings of *Streptomyces venezuelae* cells were carried out using 10 mL MSM production media without amino acid as previously described. The cells were collected and used to inoculate nine 250 mL flasks each containing 125 mL MSM production media with pH 7-7.5 using a 1 mL sterile pipette. The cultures were allowed to grow until the OD-600nm reached between 0.5-0.6 AU as recommended by Jakeman et al. (2006). This was verified using spectrophotometer (Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada).

The *S.venezuelae* culture in 3 flasks was shocked using 3% v/v anhydrous ethanol at 0 hour (control) and left for 80 h. Similarly, ethanol shock was carried out on *S.venezuelae* in another three flasks at 0 and repeated at 3 hour. The final three flasks were ethanol shocked at 0, 3 and 6 hour. All flasks were kept in environmentally controlled incubator shaker (25 Incubator Shaker, New Brunswick Scientific, Edison, New Jersey, USA) at 30°C (250 rpm).

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┌─────────────────────────────────────────┐
│ Streptomyces venezuelae spore isolated from 14 │
│           day MYM agar plates             │
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┌─────────────────────────────────────────┐      ┌──────────────────────────┐
│ Streptomyces venezuelae grown in MYM broth│─────▶│  Supernatant discarded   │
│   at 30°C, 250 rpm, and pH 7 for 18 hours │      └──────────────────────────┘
└─────────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────────┐      ┌──────────────────────────┐
│  Cells washed 3 times with MSM production │─────▶│  Supernatant discarded   │
│  media without amino acid and centrifuged at│    └──────────────────────────┘
│          4000 rpm for 10 min              │
└─────────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────────┐
│  Pellet suspended with production media at 30°C,│
│           250 rpm, and pH 7               │
└─────────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────────┐
│   Vegetative cells added to MSM production│
│  media till turbidity measurement at OD600nm│
│        reaches between 0.5-0.6 AU         │
└─────────────────────────────────────────┘
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┌──────────────────────┐   ┌──────────────────────┐   ┌──────────────────────┐
│   Control   │   │   Multiple shock - 1 │   │   Multiple shock - 2 │
│   │   │   │   │   │
│ 3% v/v ethanol at 0 h│   │   3% v/v ethanol at 0 │   │ 3% v/v ethanol at 0, 3│
│   │   │   and 3 h   │   │   and 6 h   │
└──────────────────────┘   └──────────────────────┘   └──────────────────────┘

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┌─────────────────────────────────────────┐
│  Maintained at 30°C, 250 rpm and pH adjusted│
│         after 24 h post shock             │
└─────────────────────────────────────────┘
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                    ▼
┌─────────────────────────────────────────┐
│ Samples taken at 8, 16, 24, 32, 48, 56, 64, 72 and│
│           80 h for analysis               │
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┌──────────────────────┐   ┌──────────────────────┐
│   Microbial Growth   │   │   Jadomycin   │
└──────────────────────┘   └──────────────────────┘

┌────────────────┐ ┌────────────────────────────┐ ┌────────────────────────┐ ┌──────────────────┐
│ Absorbance at │ │ Dehydrogenase Activity: │ │ Colony forming Units │ │ Absorbance at   │
│   600nm   │ │ Incubation Time: 4 hours   │ │ Serial Dilution – 10^-1 to 10^-10│ │   526nm   │
│   │ │ Incubation Temperature: 30°C│ │ Plating at 30°C for 24 hours│ │   │
│   │ │ Extraction solvent: methanol (3 time extraction)│ └────────────────────────┘ └──────────────────┘
│   │ │ Absorbance: 484nm   │
└────────────────┘ └────────────────────────────┘

Figure 4.8. Procedure for the multiple ethanol shocking experiment.

Samples (11 mL) were taken from each flask at 8, 16, 24, 32, 40, 48, 56, 64, 72 and 80 h for microbial and jadomycin analyses. 4 mL were used to measure the absorbance at 600 nm, 4 mL used to measure jadomycin absorbance at 526 nm, 2 mL were used for measuring dehydrogenase activity and 1 mL was used for plate counting.

4.4.6. *Alcohol Shock at Various Nitrogen Concentrations Experiment*

This experiment was carried out to investigate the effect of nitrogen source (L-isoleucine) at three concentrations (45, 60 and 75 mM) on the jadomycin yield at three different ethanol concentrations (3, 4.5 and 6 % v/v). The overall procedure is described in Figure 4.9. Nine 250 conical flasks were filled with 150 mL of MYM broth. The flasks were plugged with foam inserts and then covered with aluminium foil. They were autoclaved (SterileMax, Harvey/Barnstead International of Thermo Fisher Scientific, Ottawa, Ontario, Canada) at the liquid setting (121 ºC and 20 Pa) for 15 min After cooling toroom temperature, the MYM broth was inoculated with a disposable loop of *S. venezuelae* ISP5230 scraped from 14 day old MYM agar plates. The flasks were incubated in a environmentally controlled shaker (Series 25, New Brunswick Scientific Co. Inc., New Brunswick, New Jersey, U.S.) at 30 ºC with 250 rpm. The cultures were allowed to grow for 18 h.

Three samples were then taken from each flask in 50 mL centrifuge tubes and centrifuged at 4000 rpm for 10 minutes. The supernatants were discarded and cells were collected. Three washings of *Streptomyces venezuelae* were carried out using 10 mL MSM production media without amino acid. The cells were used to inoculate nine 250 mL flasks (three trials), each containing 125 mL MSM production media (pH 7-7.5) containing 45mM L-isoleucine. 1 mL sterile pipette was used to transfer 1 mL culture to each flask. The growth was observed until the OD-600nm reached between 0.5-0.6 AU as recommended by Jakeman et al. (2006). This was verified using spectrophotometer (Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada).

Three flasks were shocked with ethanol at 3% (v/v), 3 flasks were shocked with ethanol at a concentration of 4.5% (v/v) and 3 flasks were shocked with ethanol at a concentration of 6% (v/v). After the addition of alcohol, the flasks were kept in environmentally controlled incubator shaker (25 Incubator Shaker, New Brunswick Scientific, Edison, New Jersey,

Streptomyces venezuelae spore isolated from14-21 day MYM agar plates

↓

Cells washed 3 times with MSM production media without amino acid and centrifuged at 4000 rpm for 10 min → Supernatant discarded

↓

Streptomyces venezuelae grown in MYM broth at 30°C, 250 rpm, and pH 7 for 18 hours → Supernatant discarded

Pellet suspended with production media at 30°C, 250 rpm, and pH 7

↓

Vegetative cells added to MSM production media till turbidity measurement at OD600nm reaches between 0.5-0.6 AU

↓

Nitrogen Conc: 45mM (low)	Nitrogen Conc: 60mM (optimum)	Nitrogen Conc: 75mM (high)
Shock Conc: 3, 4.5 and 6% v/v ethanol	Shock Conc: 3, 4.5 and 6% v/v ethanol	Shock Conc: 3, 4.5 and 6% v/v ethanol

↓

Maintained at 30°C, 250 rpm and pH adjusted after 24 hours post shock

↓

Samples taken at 8, 16, 24, 32, 48, 56, 64, 72 and 80 h for analysis

↓

Microbial Growth

Jadomycin

Absorbance at 600nm

Dehydrogenase Activity:
Incubation Time: 4 hours
Incubation Temperature: 30°C
Extraction solvent: methanol (3 time extraction)
Absorbance: 484nm

Colony forming Units
Serial Dilution – 10^{-1} to 10^{-10}
Plating at 30°C for 24 hours

Absorbance at 526nm

Figure 4.9. Procedure for alcohol shock with varying nutrient levels experiment.

USA) at 30°C and 250 rpm. Samples (11 mL) were taken from each flask at 8, 16, 24, 32, 40, 48, 56, 64, 72 and 80 h for analysis. 4 mL were used to measure the absorbance at 600 nm, 4 mL used to measure jadomycin absorbance at 526 nm, 2 mL were used for measuring dehydrogenase activity and 1 mL was used for plate counting. The procedure was repeated with the 60 mM and 75 mM L-isoleucine concentration.

4.5. *Experimental Analysis*

4.5.1. *Absorbance Measurement for Bacterial Growth*

Three samples from the growth flasks (4 mL) were placed in disposable cuvettes (4 mL square polystyrene transparent spectrophotometer cuvettes). The absorbance was measured at 600 nm using a spectrophotometer (Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada). Pure MYM broth (without cells) was used to set the spectrophotometer to zero.

4.5.2. *Dehydrogenase Activity Measurement*

A stock solution of 0.2 μmol/mL concentration was prepared by dissolving 0.03 g triphenyl formazan in 500 mL methanol. From the stock solution, a series of 11 dilutions were prepared (0.004, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.1μmol/mL). Methanol was used as blank and the absorbance was measured at a wavelength of 484 nm (Genesys 20, Thermo Scientific, and Mississauga, Ontario, Canada). The absorbance readings are shown in Table 4.6. A standard curve was developed by plotting the absorbance of triphenyl formazan (484 nm) and concentration of triphenyl formazan (μmol/mL) as shown in Figure 4.10. The linear equation that relates the absorbance of triphenyl formazan (484 nm) and concentration of triphenyl formazan (μmol/mL) as follows ($R2$ value of 0.99):

$$OD_{484} \equiv 15.63 TF \qquad (4.1)$$

Where:

TF   is the Triphenyl formazan concentration (μmol/mL extraction solvent)

OD_{484} is the absorbance measured at 484 nm.

Table 4.6. Standard curve values relating TF concentration at absorbance at 484nm.

TF Concentration (μmol/mL)	OD$_{484nm}$
0.004	0.068
0.01	0.160
0.02	0.301
0.03	0.478
0.04	0.623
0.05	0.770
0.06	0.937
0.07	1.088
0.08	1.241
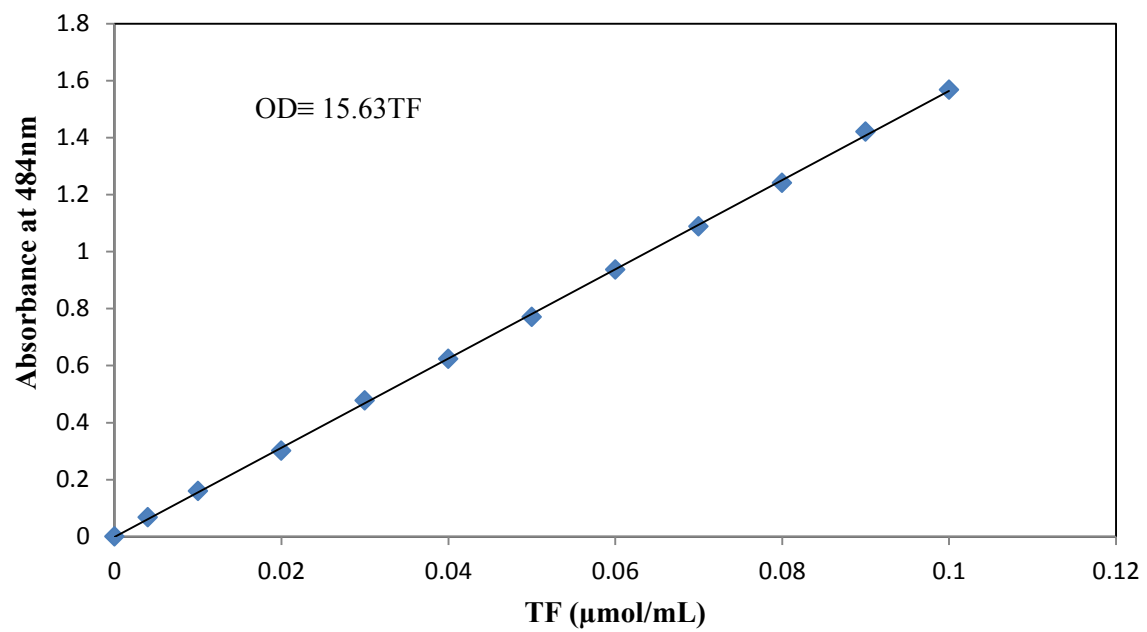
Figure 4.10. Standard curve relating TF concentration to absorbance at 484 nm.

To measure the dehydrogenase activity, 1mL of the sample was added to each of four test tubes. The pH values of these samples were noted. For the blank, 2.5 mL Tris buffer and 1mL of distilled water were added to the test tube. 1mL TTC/Glucose solution was added to other three test tubes. The pH was adjusted to 6 using 1 N NaOH or 1.0 N HCl. The test tubes were gently vortexed (Barnstead Thermolyne Type 16700 maxi-mix, Doubbuque, Iowa, USA) and swirled and then incubated at 30 ºC for 4 h. After the incubation period, the samples were centrifuged (IEC Centra CL2, Thermo Electron Corporation, Mississauga, Ontario, Canada) for 10 minutes to separate the cells from production media (Figure 4.13). The supernatant was discarded and cells were collected as pellet. Methanol (2.5 mL) was added to break the cell walls and induce the triphenyl formazan yield from the bacterial cells. Soon after the addition of methanol, the tubes were vortexed and centrifuged for 10 minutes. The supernatant was collected and 2.5 mL methanol were again added and centrifuged for 10 min to aid the second extraction. Third extraction was carried out by adding 2.5 mL methanol and centrifuged for 10 minutes. The supernatants collected from three extractions were combined together typically appearing pink colour and the absorbance was measured at 484 nm. The overall dehydrogenase measurement conditions and procedure are described in Figure 4.11.

4.5.3. *Plate Count Measurement*

A series of dilutions (10^{-1} to 10^{-10}) was carried to measure the colony forming units in MYM agar plates. 1 mL of culture sample was added to a sterile test tube (10^{-1}) containing 9 mL. The tube was vortexed (Thermolyne Maxi Mix, Thermolyne Corporation, Hampton, NH) several times to distribute the cells. An aliquot of 1 mL of this solution was transferred to a second test tube (10^{-2}). This tube was sealed and vortexed several times to distribute the cells. This procedure was carried till the final dilution of 10^{-10} was obtained. 0.1 mL was transferred to a Petri dish containing MYM agar in triplicates (for final plate dilutions of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} and 10^{-11}. The plates were sealed with Parafilm, inverted and incubated at 30ºC (Model number 2020, VWR International, Cornelius, Oregan, USA) for 24 - 36 hours. The colonies were counted using colony counter (7-910 Fisher Colony Counter, Fisher Scientific, USA). The petri plates that have 30-300 CFU were selected for calculating the cell concentration.

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                    ┌────────────────────────┐
                    │ 2.5mL Tris buffer + 1.0 mL │
                    │ TTC/glucose solution + 1mL │
                    │         sample          │
                    └────────────────────────┘
                                │
                    ┌────────────────────────┐
                    │ Incubate at pH 6, 30°C for 4 │
                    │          hour           │
                    └────────────────────────┘
                                │
                    ┌────────────────────────┐        ┌────────────────────────┐
                    │ Centrifugation at 4000rpm │───────▶│  Supernatant Discarded  │
                    │        for10 min        │        └────────────────────────┘
                    └────────────────────────┘
                                │
                      ┌──────────────────┐
                      │  Pellet Collected │
                      └──────────────────┘
                                │
                    ┌────────────────────────┐
                    │  First Extraction using 2.5 ml │
                    │         methanol        │
                    └────────────────────────┘
                                │
                    ┌────────────────────────┐        ┌────────────────────────┐
                    │ Vortexed and Centrifuged at 4000 │──────▶│   Supernatant Saved    │
                    │      rpm for 10 min      │        └────────────────────────┘
                    └────────────────────────┘
                                │
                      ┌──────────────────┐
                      │ Pellets Collected │
                      └──────────────────┘
                                │
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                    │ Second Extraction using 2.5 ml │
                    │         methanol        │
                    └────────────────────────┘
                                │
                    ┌────────────────────────┐        ┌────────────────────────┐
                    │ Vortexed and Centrifuged at 4000 │──────▶│   Supernatant Saved    │
                    │      rpm for 10 min      │        └────────────────────────┘
                    └────────────────────────┘
                                │
                      ┌──────────────────┐
                      │ Pellets Collected │
                      └──────────────────┘
                                │
                    ┌────────────────────────┐
                    │  Third Extraction using 2.5 ml │
                    │         methanol        │
                    └────────────────────────┘
                                │
 ┌──────────────────┐ ┌────────────────────────┐        ┌────────────────────────┐
 │ Pellets Discarded │◀─│ Vortexed and Centrifuged at 4000 │──────▶│   Supernatant Saved    │
 └──────────────────┘ │      rpm for 10 min      │        └────────────────────────┘
                    └────────────────────────┘
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Combined Supernatant Absorbance measured at 484nm

Figure 4.11. Procedure for Dehydrogenase measurement during growth and production of *Streptomyces venezuelae* ISP 5230 VS1099.

To determine the number of colony forming units per mL;

$$CFU/mL = \frac{\text{Number of CFU (average of 3 replicates)}}{\text{Volume plated (mL) x total dilution used}} \qquad (4.2)$$

4.5.4. *Jadomycin Measurement*

According to Jakeman et al (2006) and Burdock et al (2008), jadomycin can be determined by measuring the distinct dark reddish color (Figure 4.12) formed during the shocking experiment. Sample (4 mL) obtained from three production trials were placed in 50 mL centrifuge tubes (Corning Centrifuge Tubes, Falcon) and centrifuged at 4000 rpm for 10 minutes. The reddish supernatants were poured in disposable cuvettes (4 mL square polystyrene transparent spectrophotometer cuvettes) and then the absorbance was measured at 526 nm using a spectrophotometer (Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada). Pure MSM production media was used to set the spectrophotometer to zero.

Figure 4.12. Separation of cells from production medium by centrifugation showing dark reddish colour of jadomycin B.

CHAPTER 5. RESULTS

5.1. *Streptomyces venezuelae Growth*

Streptomyces venezuelae was grown in MYM broth medium for 65 h in an environmentally controlled shaker at 30° C and 250 rpm. The growth of *Streptomyces venezuelae* ISP5230 was determined by measuring the absorbance, number of colony forming units count (CFU) and dehydrogenase activity at several time intervals. The data are shown in Table A.1 (Appendix A) and the results are presented in Figures 5.1-5.3.

The relationships between the absorbance OD 600nm and CFU/mL and between absorbance OD 600nm and TF yield are shown in Figures 5.4 and 5.5 respectively. The relationship between the TF yield and CFU was much better than that between absorbance (OD 600nm) and the TF yield. This was probably due to the fact that absorbance measures both living and the dead cells, while TF yield and CFU measure only living cells.

The lag period and specific growth rate of *Streptomyces venezuelae* were determined graphically using the CFU data (Figure 5.6) according to the procedure described by Nair (2007) and Widdel (2010). A lag period of 6 h was observed. This was followed by an exponential growth period that lasted from the 6th h to 50th h from the time of inoculation. The specific growth rate (μ) was 0.19 h^{-1}. Following the exponential growth phase, a stationary growth period was observed from the 50th h till the end of the experiment. After 50 h of growth in MYM nutrient rich broth (end of exponential period), the *S.venezuelae* reached an average cell concentration of 1.92 x 10^{-10} cells per mL. The corresponding absorbance reading at 600 nm was 2.132 AU and the triphenyl formazan yield (TF) was 0.847 μmol. However, the cell concentration of *S.venezuelae* remained approximately constant during the stationary period. The corresponding absorbance reading at 600 nm was 2.128 AU and the triphenyl formazan yield (TF) was 0.828 μmol.

The specific activity (μmol/CFU) was calculated by dividing the TF yield by the number of CFU in order to determine the cell activity during the various growth periods. The results are shown in Figure 5.7. The results indicated that the activity of *S.venezuelae* during
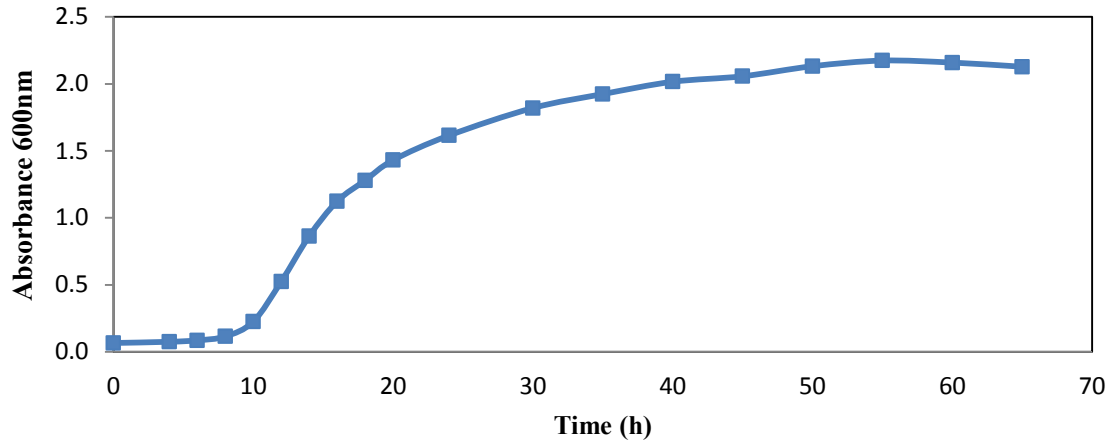
Figure 5.1. The growth of *S. venezuelae* in MYM broth measured as absorbance 600 nm.
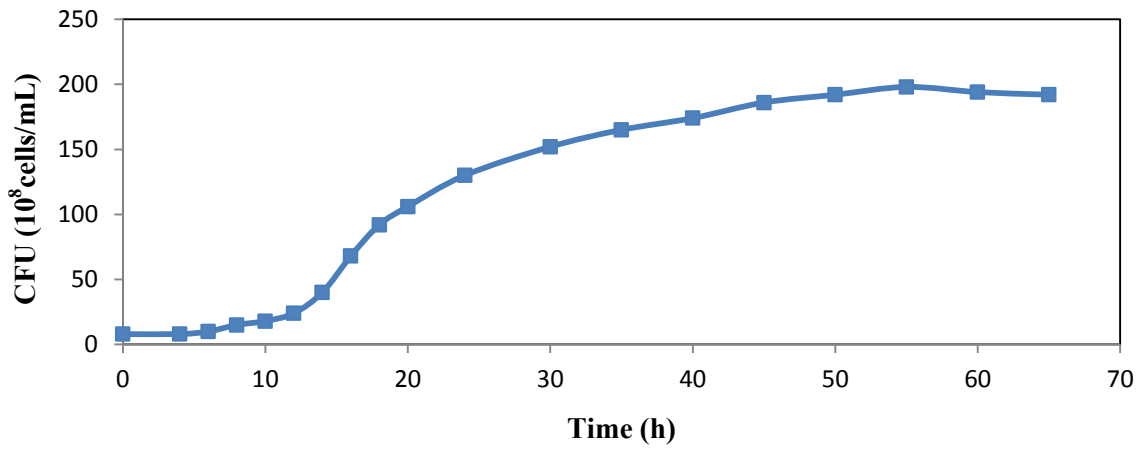
Figure 5.2. The growth of *S.venezuelae* in MYM broth measured as colony forming units (CFU).
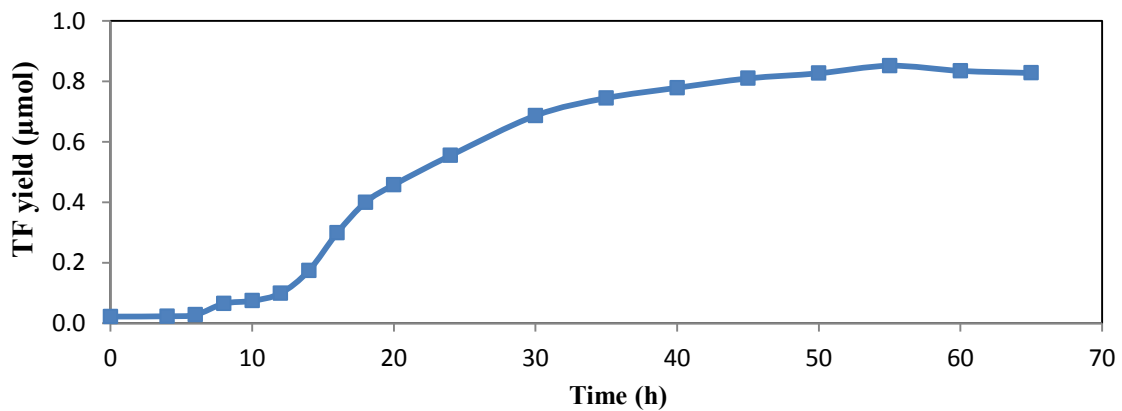
Figure 5.3. The growth of *S. venezuelae* in MYM broth measured as triphenyl formazan yield (TF).
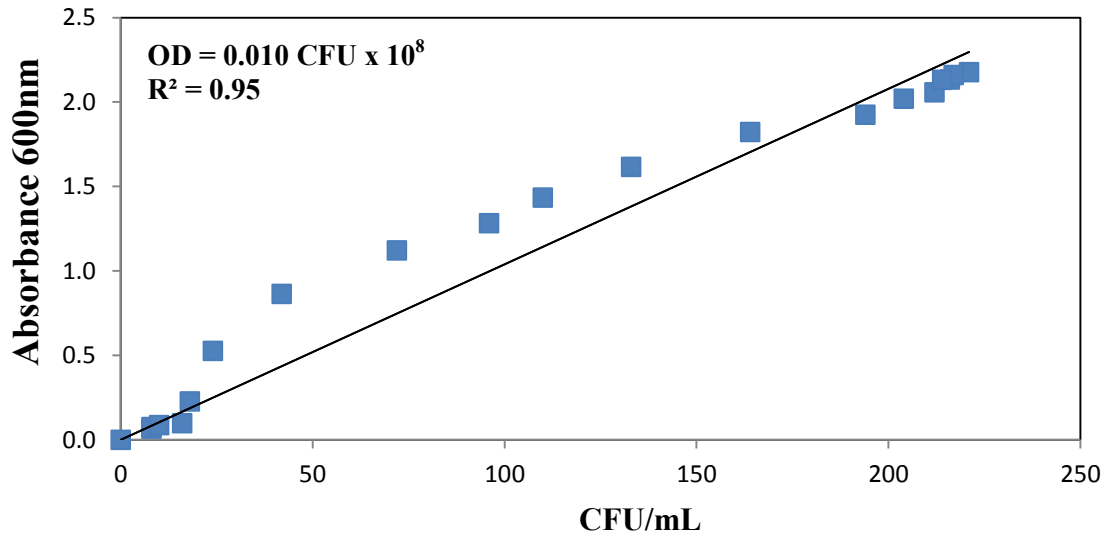
Figure 5.4. The relationship between optical density (OD) and CFU.
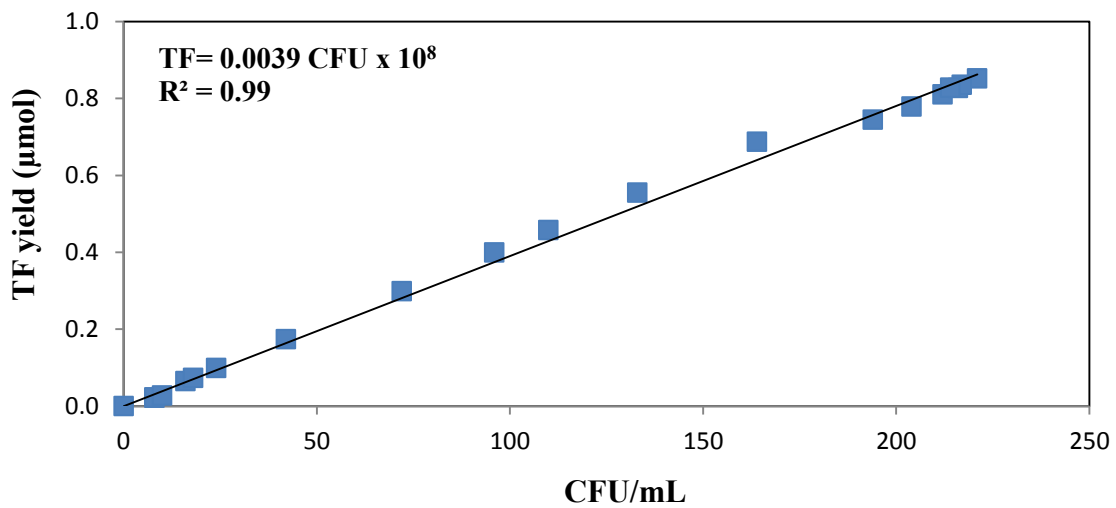
Figure 5.5. The relationship between TF yield and CFU.
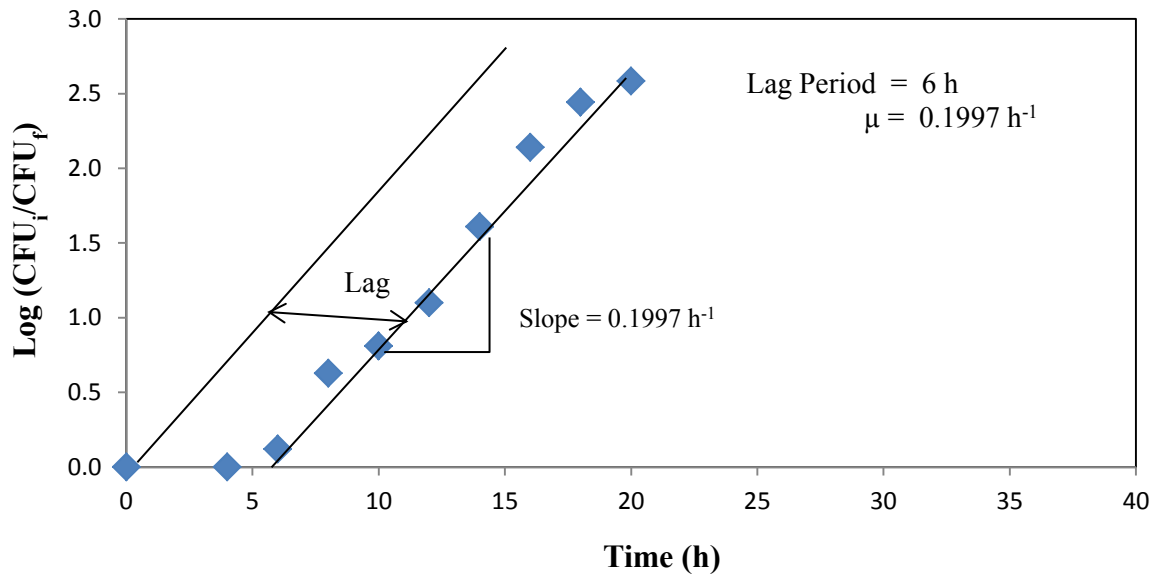
Figure 5.6. Graphical determination of the lag period and specific growth rate.
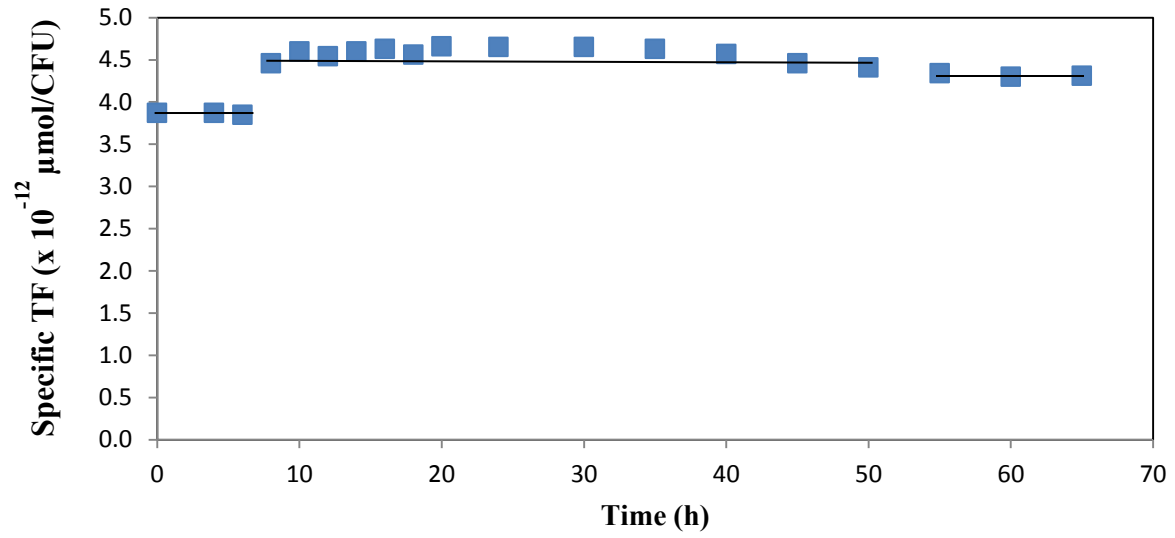
Figure 5.7. The activity of *S.venezuelae* as measured by specific TF during growth in MYM growth media.

the lag phase (0 - 6 h), exponential growth phase (6 - 50 h) and stationary phase (55 - 65 h) were 3.80×10^{-11}, 4.41×10^{-11} and 4.31×10^{-11} μmol/CFU respectively. The specific TF yield remained constant during the entire growth period indicating the ability of TF as a measure of *S.venezuelae* activity.

5.2. *Time of Culture Transfer into Production Medium*

The effect of the time of transferring the cells (18, 21 and 24 h) from the growth medium to the production medium on the growth of *S.venezuelae* and production of jadomycin was evaluated. Jadomycin production was initiated by shocking the healthy *S.venezuelae* cells after transfer to the nutrient deprived amino acid rich MSM production media using 3% v/v ethanol (pH of 7). The dehydrogenase activity (measured through triphenyl formazan (TF) yield), colony forming units and jadomycin B production were determined at several time intervals. The data are shown in Table B.1 (Appendix B).

5.2.1. *Streptomyces venezuelae Growth*

Figures 5.8 and 5.9 show the CFU and TF yield measured at time intervals. At the time of inoculations in the nutrient deprived amino acid rich MSM production media, the CFU was 0.21×10^8, 0.21×10^8 and 0.20×10^8 CFU/mL and the corresponding triphenyl formazan yield was 0.028, 0.028 and 0.026 μmol for the cultures transferred after 18, 21 and 24 h, respectively. The cell number increased with time reaching 1.42×10^8, 1.38×10^8 and 1.36×10^8 CFU/mL at the 32 hour for the culture transfered after 18, 21 and 24 h, respectively. The TF yield increased to 0.452, 0.433 and 0.412 μmol at the 32 h for the cultures transferred after 18, 21 and 24 h, respectively. The cell growth then declined after the first 32 h reaching 1.14×10^8, 1.12×10^8 and 1.04×10^8 CFU/mL at the end of the experiment (72 h) for the cultures transferred after 18, 21 and 24 h, respectively. The corresponding final triphenyl formazan (TF) yield was 0.155, 0.146 and 0.125 μmol for the culture transferred after 18, 21 and 24 h, respectively. The results indicated that culture transferred to the production medium after 18 hour of growth in the growth medium resulted in higher cell number and TF yield during the entire period of growth and jadomycin production in the MSM production medium.

Figure 5.8. The growth of various *S.venezuelae* cultures in MSM production media measured as colony forming units (CFU).
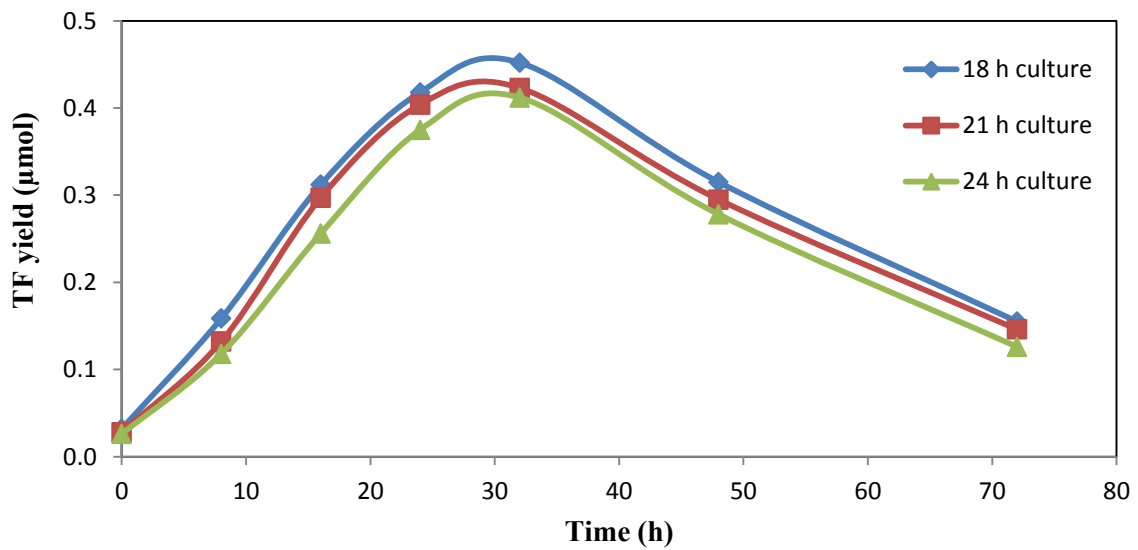
Figure 5.9. The activity of various *S.venezuelae* cultures in MSM production media measured as triphenyl formazan yield (TF).

Analyses of variance (ANOVA) were performed on the TF yield data using Minitab (Quality Plaza, State College, PA, USA) as shown in Tables 5.1. The results showed that the effects of time of transferring the culture from the growth medium to the production medium and the time of samples from production medium were significant at the 0.001 level. There was a significant interaction between the time of transferring the culture and the sampling timeat the 0.001 level.

The results obtained from the Tukey grouping are shown in Tables 5.2. The levels of the culture transfer time (18, 21 and 24 hour) were significantly different from one another at the 0.05 level. The highest average TF yield of 0.302% was achieved at the culture transfer time of 18 h. Also all levels of sampling time were significantly different from each other at the 0.05 level. The highest TF yield was produced at the 32 h.

5.2.2. *Streptomyces venezuelae Specific Activity*

The specific activity (μmol/CFU) was used in this study to assess the level of *Streptomyces venezuelae* activity while producing jadomycin in the production medium. The specific activity was calculated by dividing the TF yield by the CFU. The results are shown in Figure 5.10. At the time of inoculation in the nutrient deprived amino acid rich MSM production media, the specific activity was 1.33×10^{-9}, 1.33×10^{-9} and 1.30×10^{-9} μmol/CFU for the culture transferred after 18, 21 and 24 h, respectively. Both the cell number and TF yield increased with time reaching their maximum values at the 32 h. However, the TF increased at a faster rate than that of the cells during the first 32 h, resulting in an increased specific activity. The highest specific activity was 3.18×10^{-9}, 3.13×10^{-9} and 3.02×10^{-9} μmol/CFU for the cultures transferred after 18, 21 and 24 h, respectively. After 32 h, the TF yield declined at a faster rate than that of the cells and as a result, the specific activity slightly declined to 1.35×10^{-9}, 1.30×10^{-9} and 1.21×10^{-9} μmol/CFU at the end of the experiment (72 h) for the cultures transferred after 18, 21 and 24 h, respectively.

5.2.3. *Jadomycin Production*

Jadomycin B production was monitored by measuring the absorbance (AU 526) at the wavelength of 526nm as suggested by Jakeman et al (2006). Immediately after the ethanol shock at 0 h, formation of dark reddish orange color was noted for all transfer cultures.

Table 5.1. Analysis of variance for TF yield data.

Source	DF	SS	MS	F	P
Total	53	0.734555			
Model					
Transfer Time	2	0.027761	0.013881	253.66	0.001
SamplingTime	5	0.686857	0.137371	2510.34	0.001
Sampling Time * Transfer Time	10	0.017967	0.001897	32.83	0.001
Error	36	0.001970	0.000055		

DF : Degree of Freedom
SS   : Sum of Square
MS : Mean of Square
R^2 : 0.997
CV : 4.29%

Table 5.2. Tukey grouping for TF Yield data.

Factors	Level	N	Mean Yield % (μmol)	Grouping
	18	18	0.302	A
Transfer time (h)	21	18	0.272	B
	24	18	0.247	C
	8	9	0.132	A
Sampling Time (h)	16	9	0.303	B
	24	9	0.385	C
	32	9	0.420	D
	48	9	0.275	E
	72	9	0.127	F

Groups with the same letter are not significantly different from each other at the 0.05 level.
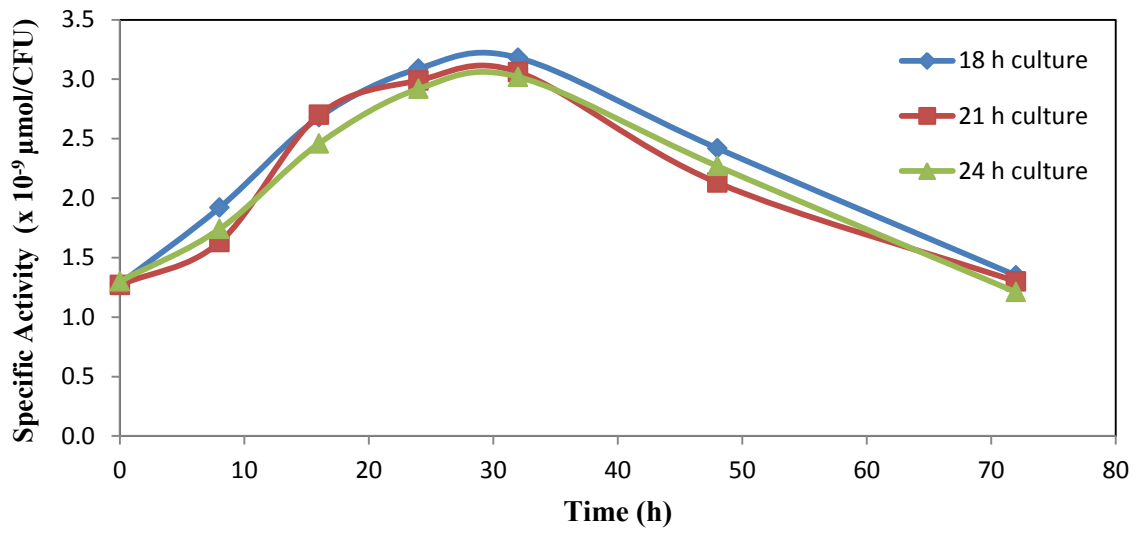
Figure 5.10. The specific activity of various *S.venezuelae* cultures as measured by specific TF in MSM production media.

The jadomycin B production [absorbance (AU 526)] was noted to increase slowly during the first 8 h, followed by a rapid increase reaching 0.394 AU, 0.423 AU and 0.430 AU at 72 h for the cultures transferred after 18, 21 and 24 h, respectively (Figure 5.12).

Analyses of variance (ANOVA) were performed on the jadomycin yield data using Minitab (Quality Plaza, State College, PA, USA) as shown in Tables 5.3. The results showed that the effects of the culture transfer time and sampling time on jadomycin production were significant at the 0.001 levels. The results also showed a significant interaction between the transfer time and sampling time at the 0.001 level.

The results obtained from the Tukey grouping are shown in Tables 5.4. The 21 and 24 h culture transfer times were not significantly different from one another at the 0.05 level, but were significantly different from the 18 h transfer time at the 0.05 level. The highest jadomycin was produced by the culture that was transferred after 18 h from the growth medium to the production medium. All the levels of sampling times were significantly different from one another at the 0.05 level. The highest jadomycin production was produced after 72 h.

5.3. *Heat Shock in Production Medium*

Since the jadomycin B production was previously shown to be associated with growth of *S.venezuelae* at high temperatures, experiments were carried out to determine the optimum temperature for the production of this secondary metabolite. Various heat shock treatments (35. 40, 45 and 50°C) at two exposure times (0.5 and 1 h) of the *S.venezuelae* in the nutrient deprived amino acid rich MSM production media were evaluated. The dehydrogenase activity (measured through triphenyl formazan (TF) yield), colony forming units (CFU) and jadomycin B production were determined at several time intervals. The data are shown in Tables C.1–C.4 (Appendix C).

5.3.1.*Streptomyces venezuelae Growth*

Figures 5.13 - 5.16 show the effects of temperature and exposure time on the growth of *S.venezuelae* measured as CFU and TF yield at time intervals. At the time of inoculations in
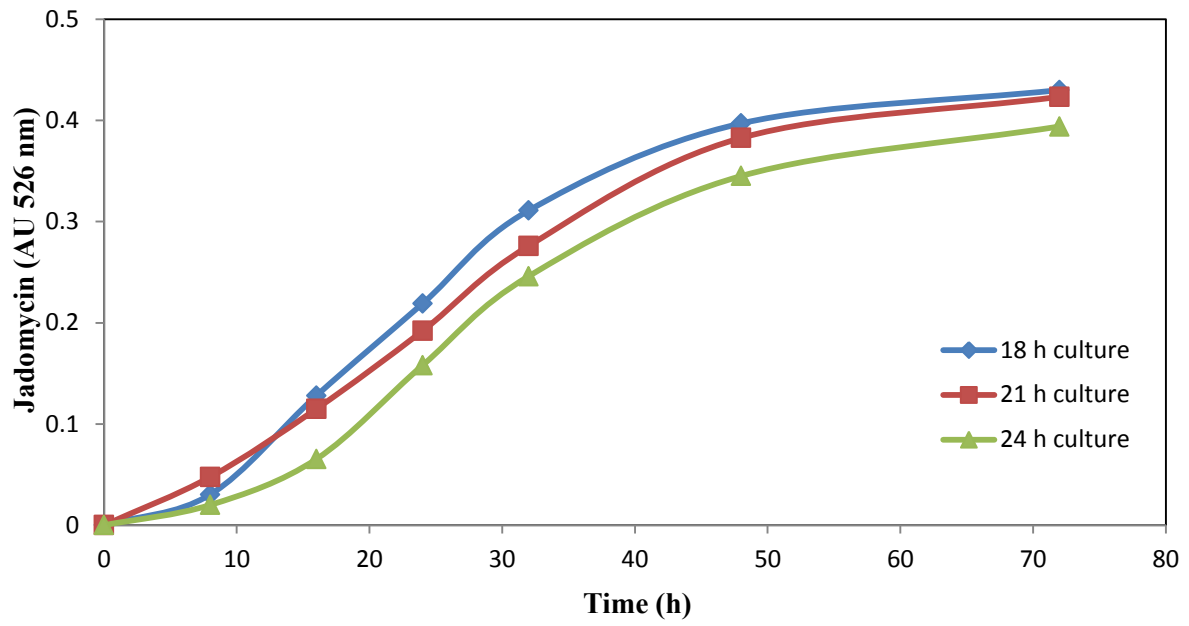
Figure 5.11. Jadomycin production measured at optical density in MSM production media.

Table 5.3. Analysis of variance for jadomycin production data.

Source	DF	SS	MS	F	P
Total	53	1.040095			
Model					
Transfer Time	2	0.0298835	0.014918	42.92	0.001
Sampling Time	5	0.991461	0.198292	570.56	0.001
Sampling Time * Transfer Time	10	0.006287	0.006629	17.81	0.001
Error	36	0.18673	0.000519		

DF : Degree of Freedom
SS : Sum of Square
MS : Mean of Square
R^2 : 0.988
CV : 6.10%

Table 5.4. Tukey grouping on jadomycin production data.

Factors	Level	N	Mean Yield % (AU_{526nm})	Grouping
Transfer time (h)	18	18	0.252	A
	21	18	0.237	A
	24	18	0.197	B
Sampling Time (h)	8	9	0.032	A
	16	9	0.102	B
	24	9	0.186	C
	32	9	0.278	D
	48	9	0.365	E
	72	9	0.410	F

Groups with the same letter are not significantly different from each other at the 0.05 level
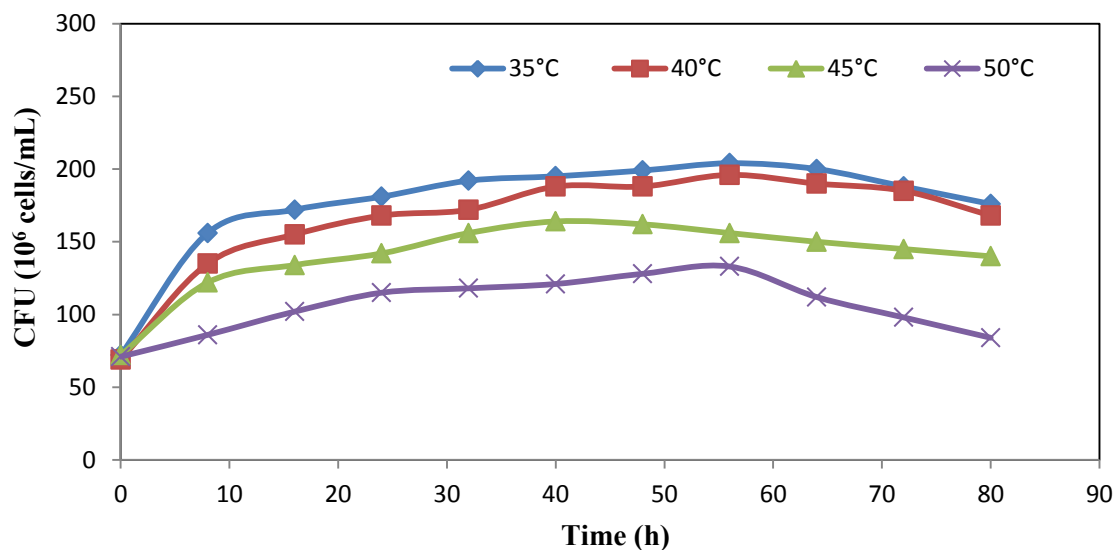
Figure 5.12. Effect of heat exposure for 0.5 h on the growth of *S.venezuelae* in MSM production media measured as colony forming units (CFU).
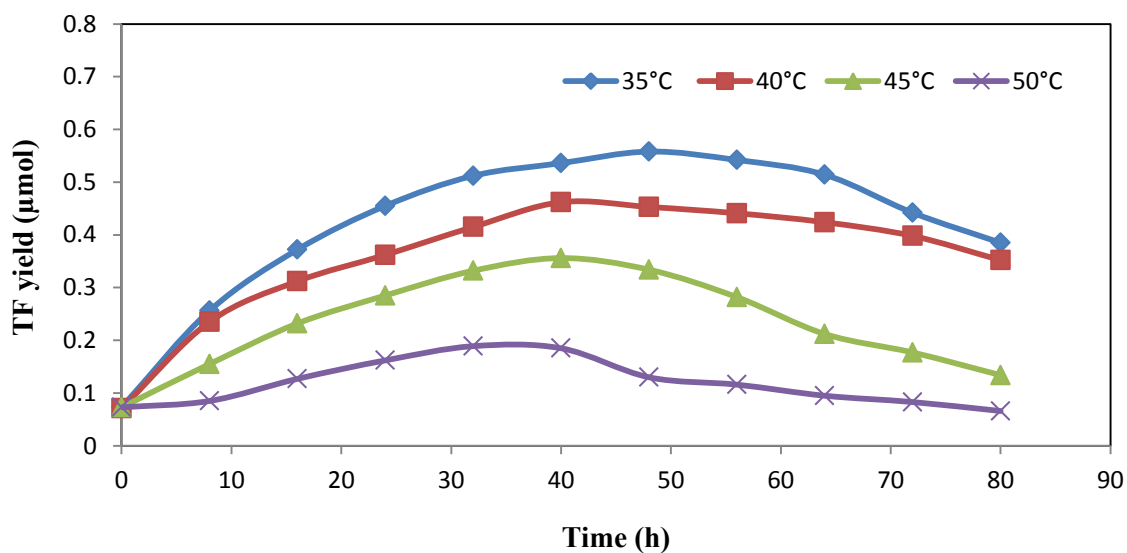
Figure 5.13. Effect of heat exposure for 0.5 h on the activity of *S.venezuelae* in MSM production media measured as triphenyl formazan yield (TF).
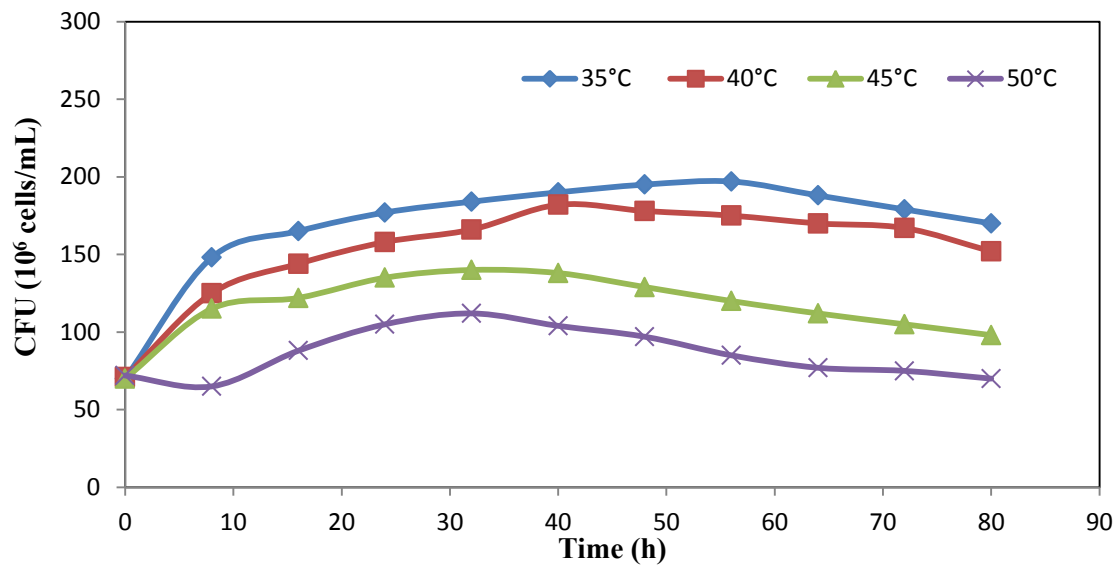
Figure 5.14. Effect of heat exposure for 1 h on the growth of *S.venezuelae* in MSM production media measured as colony forming units (CFU).
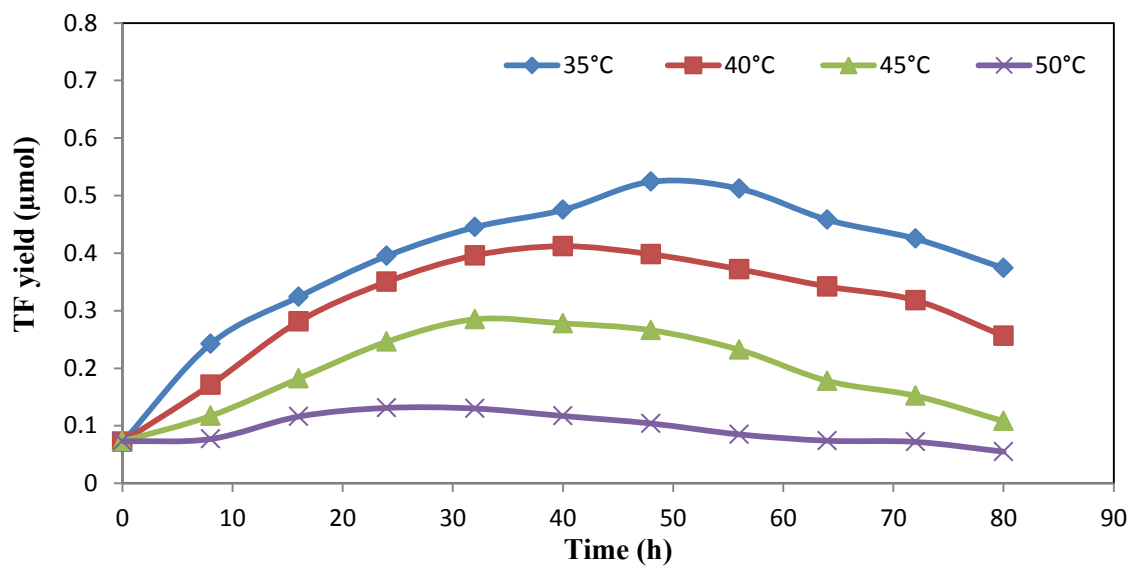
Figure 5.15. Effect of heat exposure for 1 h on the activity of *S.venezuelae* in MSM production media measured as triphenyl formazan yield (TF).

the nutrient deprived amino acid rich MSM production media, the CFU/mL for the 0.5 h exposure time was 0.72 x 10^8, 0.69 x 10^8, 0.72 x 10^8 and 0.71 x 10^8 CFU/mL and the corresponding triphenyl formazan yield was 0.073, 0.071, 0.073 and 0.073 μmol for the cultures that were heat treated at the temperatures of 35, 40, 45 and 50°C, respectively. For the 1 h exposure time, the CFU was 0.70 x 10^8, 0.71 x 10^8, 0.70 x 10^8 and 0.72 x 10^8 CFU/mL and the corresponding triphenyl formazan yield was 0.071, 0.072, 0.073 and 0.073 μmol for the cultures that were heat treated at the temperatures of 35, 40, 45 and 50°C, respectively.

The cell number increased with time reaching a maximum of 2.04 x 10^8 CFU/mL at 56 h, 1.96 x 10^8 CFU/mL at 56 h, 1.64 x 10^8 CFU/mL at 40 h and 1.3 3 x 10^8 CFU/mL at the 56 hour for the cultures exposed to the heat treatment for 0.5 h at the temperatures of 35, 40, 45 and 50°C, respectively. The corresponding TF yield also increased to a maximum of 0.558 μmol at 48 h, 0.462 μmol at 40 h, 0.356 μmol at 40 h and 0.189 μmol at 32 h for the cultures exposed to heat treatment for 0.5 exposed to the temperatures of 35, 40, 45 and 50°C, respectively.

When the cultures were exposed to heat treatment for 1 h, the cell number increased with time to a maximum of 1.97 x 10^8 CFU/mL at 56 h, 1.82 x 10^8 CFU/mL at 40 h, 1.40 x 10^8 CFU/mL at 32 h and 1.12 x 10^8 CFU/mL at the 32 h for the culture exposed to the temperatures of 35, 40, 45 and 50°C, respectively. The corresponding TF yield also increased to a maximum of 0.524 μmol at 48 h, 0.412 μmol at 40 h, 0.285 μmol at 32 h and 0.131 μmol at 24 h for the cultures exposed to the temperatures of 35, 40, 45 and 50°C, respectively.

The cell number then declined reaching 1.76 x 10^8, 1.68 x 10^8, 1.40 x 10^8 and 0.84 x 10^8 CFU per mL at the end of the experiment (80 h) for the cultures exposed to heat shock for 0.5 h at the temperatures of 35, 40, 45 and 50°C, respectively. The corresponding final triphenyl formazan (TF) yield was 0.385, 0.352, 0.134 and 0.066 μmol for the culture exposed to heat shock for 0.5 h at the temperatures of 35, 40, 45 and 50°C, respectively.

For the heat shocked cultures for 1 h, the cell number declined reaching 1.70 x 10^8, 1.52 x 10^8, 0.98 x 10^8 and 0.70 x 10^8 at end of the experiment (80 h) for the cultures exposed to the temperatures of 35, 40, 45 and 50°C, respectively. The corresponding final triphenyl

formazan (TF) yield was 0.374, 0.256, 0.108 and 0.055 µmol for the culture exposed to heat shock for 1 h at the temperatures of 35, 40, 45 and 50°C, respectively.

The results indicated that the highest cell number and highest TF were not reached at the same time for all treatments. However, the culture heat shocked at 35˚C for 0.5 h exposure time in the production medium resulted in higher cell number and TF yield during the entire period of growth in the MSM production medium.

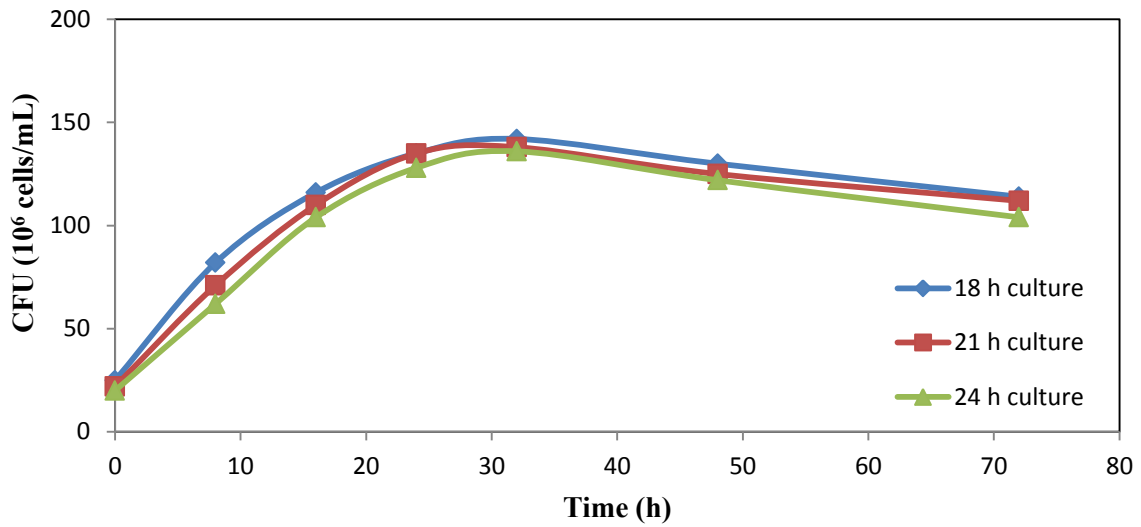Analyses of variance (ANOVA) were performed on the TF yield data using Minitab (Quality Plaza, State College, PA, USA) as shown in Tables 5.5. The results showed that the effects of temperature, exposure time and sampling time were significant at the 0.001 level. There were significant interactions between the heat shock temperature, exposure time and the sampling time at the 0.001 level.

The results obtained from the Tukey grouping are shown in Tables 5.6. The levels of temperature were significantly different from one another at the 0.05 level. The highest average TF yield of 0.439 µmol was achieved by the culture heat shocked at 35˚C. The levels of exposure time were significantly different from one another at the 0.05 level. The highest average TF yield of 0.297 µmol was achieved at the exposure time of 0.5 h. All the levels of sampling times were significantly different from one another at the 0.05 level. The highest averageTF yield of 0.344 µmol was produced at the 48 h.

5.3.2. *Streptomyces venezuelae Specific Activity*

The specific activity results are shown in Figures 5.17 and 5.18. At the time of inoculation in the nutrient deprived amino acid rich MSM production media, the specific activity was 1.01×10^{-9}, 1.02×10^{-9}, 1.01×10^{-9} and 1.02×10^{-9} µmol/CFU for the cultures exposed to heat shock for 0.5 h at the temperatures of 35, 40, 45 and 50°C, respectively. The initial specific TF was 1.01×10^{-9}, 1.01×10^{-9}, 1.04×10^{-9} and 1.01×10^{-9} µmol/CFU for the cultures exposed to heat shock for 1 h at the temperatures of 35, 40, 45 and 50°C, respectively. Both the cell number and TF yield increased with time. However, the TF yield increased at a faster rate than that of the cell number in the first 40 h, resulting in increase in the specific activity. The highest specific activity was 2.73×10^{-9}, 2.35×10^{-9}, 2.17×10^{-9} and 1.42×10^{-9} µmol/CFU for the cultures exposed to heat shock for 0.5 h at the temperatures of

Table 5.5. Analysis of variance for TF yield data.

Source	DF	SS	MS	F	P
Total	239	5.177957			
Model					
Temperature	3	4.061180	135372	26285.95	0.001
Exposure Time	1	0.068716	0.068716	1334.28	0.001
Sampling Time	9	0.760321	0.084480	1640.39	0.001
Temperature*Exposure Time	3	0.024862	0.08284	160.92	0.001
Temperature*SamplingTime	27	0.234405	0.008672	168.58	0.001
Exposure Time*Sampling Time	9	0.002489	0.000277	5.37	0.001
Temperature*Exposure Time*SamplingTime	27	0.017745	0.000658	12.76	0.001
Error	160	0.008240	0.000051		

DF : Degree of Freedom
SS   : Sum of Square
MS : Mean of Square
R^2 : 0.998
CV : 5.24%

Table 5.6. Tukey grouping for TF Yield data.

Factors	Level	N	Mean % (μmol)	Grouping
Temperature (°C)	35	60	0.439	A
	40	60	0.357	B
	45	60	0.230	C
	50	60	0.096	D
Exposure Time (h)	0.5	120	0.297	A
	1.0	120	0.263	B
SamplingTime (h)	8	24	0.164	A
	16	24	0.241	B
	24	24	0.290	C
	32	24	0.329	D
	40	24	0.342	E
	48	24	0.344	F
	56	24	0.317	G
	64	24	0.268	H
	72	24	0.259	I
	80	24	0.220	J

Groups with the same letter are not significantly different from each other at the 0.05 level.
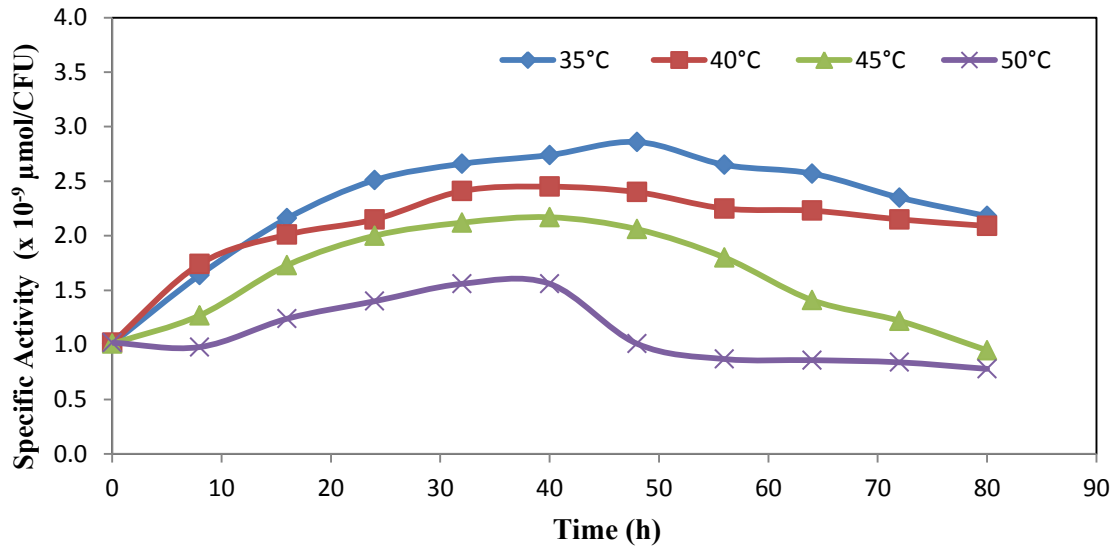
Figure 5.16. The activity of *S.venezuelae* cultures exposed to various heat shocks for 0.5 h as measured by specific TF yield in the MSM production media.
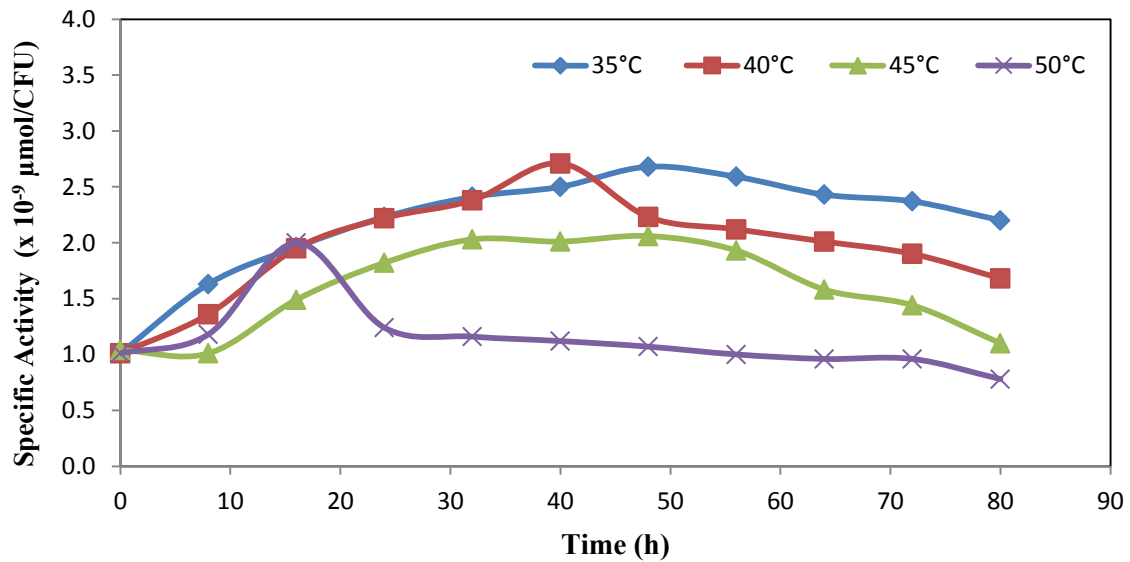
Figure 5.17. The specific activity of *S.venezuelae* cultures exposed to various heat shocks for 1 h as measured by specific TF yield in the MSM production media.

35, 40, 45 and 50°C, respectively. Similarly, for the cultures exposed to heat shock for 1 h, the specific TF increased reaching 2.65×10^{-9}, 2.26×10^{-9}, 2.03×10^{-9} and 1.16×10^{-9} μmol/CFU for the cultures exposed to heat shock at the temperatures of 35, 40, 45 and 50°C, respectively.

After reaching the maximum growth, the decline in the TF yield was faster than that of the cell number and as a result the specific TF slightly declined. The final specific activity was 2.18×10^{-9}, 2.09×10^{-9}, 0.95×10^{-9} and 0.78×10^{-9} μmol/CFU at the end of the experiment (at 80 h) for the cultures exposed to heat shock for 0.5 h at the temperature 35, 40, 45 and 50°C, respectively. Similarly, the final specific activity for the cultures exposed to heat shock for 1 h was 2.20×10^{-9}, 1.68×10^{-9}, 1.10×10^{-9} and 0.78×10^{-9} μmol/CFU at the temperatures of 35, 40, 45 and 50°C, respectively.

5.3.3. *Jadomycin Production*

Jadomycin B was successfully produced as was indicated by the increase in absorbance (AU 526nm) and the change in color of production medium to dark reddish orange. The jadomycin B production [absorbance (AU 526)] was noted to increase slowly during the first 24 h for heat shocked cultures at the temperatures of 35, 45 and 50°C for 0.5 h followed by a rapid increase (Figure 5.19) thereafter. However, the culture that was heat shocked at 40°C started to produce significant amount of jadomycin after 10 h. The final jadomycin amount was 0.042, 0.235, 0.328 and 0.155 AU for the cultures heat shocked for 0.5 h at the temperatures of 35, 40, 45 and 50 °C, respectively.

Similarly, for the 1 h exposure time (Figure 5.20), the jadomycin B production [absorbance (AU 526)] was noted to increase slowly during the first 16 h for the cultures exposed to heat shock at the temperatures of 35 and in 50°C. However, the cultures exposed to heat shock at the temperatures of 40 and 45°C started to produce jadomycin immediately after shocking. The final absorbance of jadomycin (at 80 h) was 0.061, 0.258, 0.282 and 0.135 AU for the cultures heat shocked for 1 h at the temperatures of 35, 40, 45 and 50 °C, respectively.

Analyses of variance (ANOVA) were performed on the jadomycin yield data using Minitab (Quality Plaza, State College, PA, USA) as shown in Tables 5.7. The results
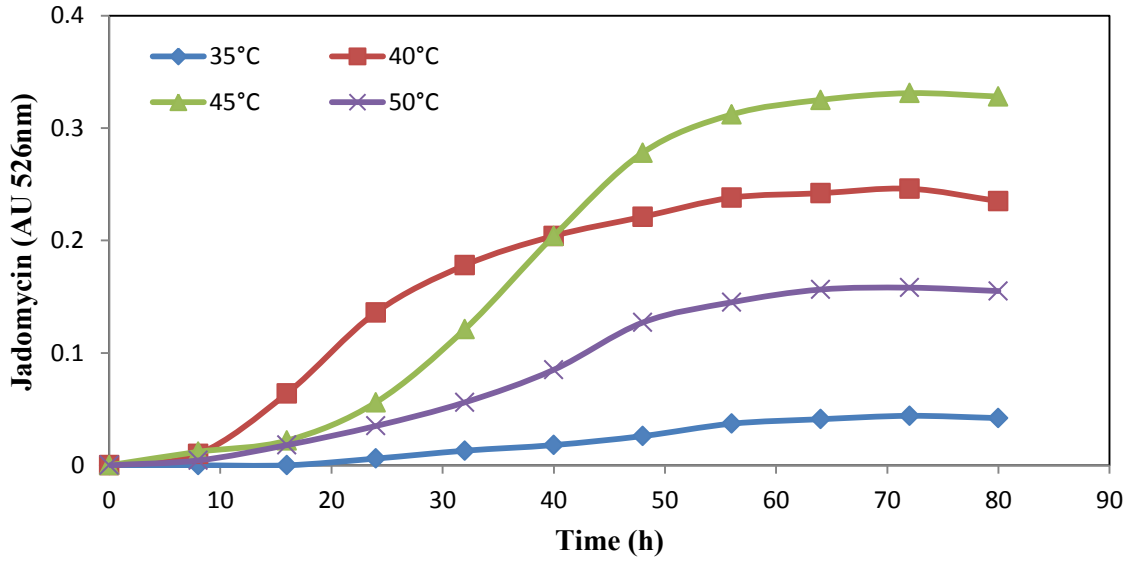
Figure 5.18. Jadomycin production measured as optical density for various heat shock temperatures with exposure of 0.5 h in the MSM production media.
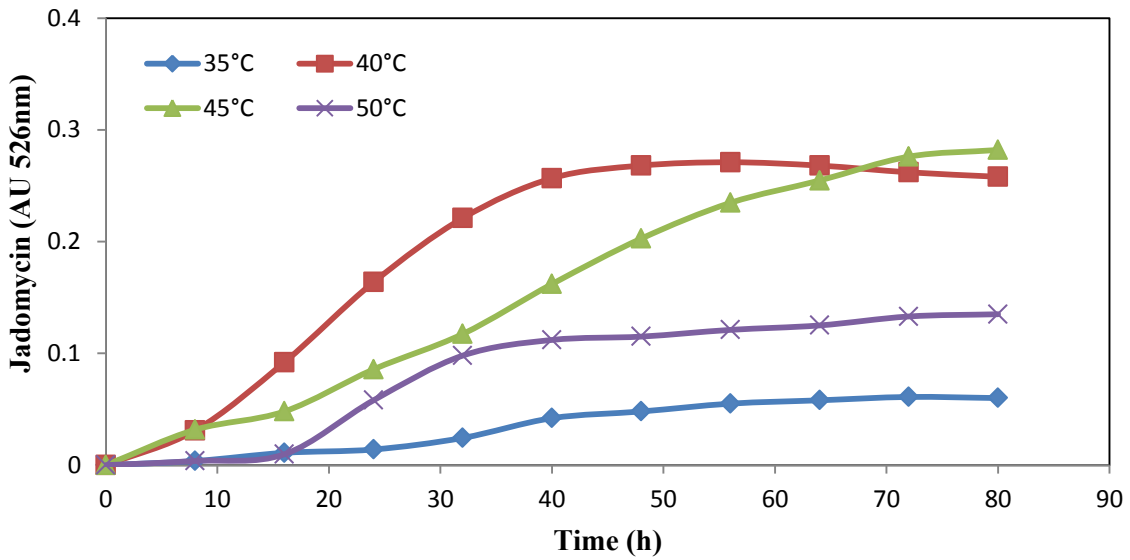
Figure 5.19. Jadomycin production measured as optical density for various heat shock temperatures with exposure of 1 h in the MSM production media.

Table 5.7. Analysis of Variance for jadomycin production data.

Source	DF	SS	MS	F	P
Total	239	2.388469			
Model					
Temperature	3	1.093531	0.364510	4001.39	0.001
Exposure Time	1	0.002227	0.002237	24.44	0.001
SamplingTime	9	0.920524	0.102280	1122.78	0.001
Temperature*Exposure Time	3	0.034500	0.011504	126.24	0.001
Temperature*Sampling Time	27	0.281657	0.010432	114.51	0.001
Exposure time* SamplingTime	9	0.018073	0.002008	22.04	0.001
Temperature*Exposure Time*SamplingTime	27	0.023384	0.000866	9.51	0.001
Error	160	0.014575	0.000091		

DF : Degree of Freedom
SS   : Sum of Square
MS : Mean of Square
R^2 : 0.993
CV : 7.85%

showed that the effects of heat shock temperature, exposure time and sampling time on jadomycin production were significant at the 0.001 level. The results also showed significant interactions between the heat shock temperature, exposure time and sampling time at the 0.001 level.

The results obtained from the Tukey grouping are shown in Tables 5.8. The levels of temperature were significantly different from one another at the 0.05 level. The highest jadomycin (0.191 AU) was produced by the culture that was heat shocked at a temperature of 40°C. The two exposure times were significantly different from one another. The exposure time of 0.5 h produced the highest jadomycin (0.132 AU). All the levels of sampling time were significantly different from one another at the 0.05 level. The highest jadomycin production (0.191 AU) was observed at the 80 h.

5.4. *Alcohol Shock in Production Medium*

Experiments were carried out to determine the most effective alcohol and the optimum alcohol concentration for shocking *S.venezuelae* for production of the secondary metabolite jadomycin. Two alcohol types (ethanol and methanol) and four alcohol concentrations (3.0, 4.5, 6.0 and 7.5 % v/v) were evaluated. The colony forming units, dehydrogenase activity (measured through triphenyl formazan (TF) yield) and jadomycin B production were determined at several time intervals. The data are shown in Tables D.1–D.4 (Appendix C).

5.4.1. *Streptomyces venezuelae Growth*

Figures 5.21 - 5.24 show the effects of ethanol and methanol concentrations on the growth of *S.venezuelae* measured as CFU and TF yield at various time intervals. At the time of inoculation in the nutrient deprived amino acid rich MSM production media, the CFU for the cultures to be shocked with ethanol was 0.62×10^8, 0.65×10^8, 0.63×10^8 and 0.62×10^8 CFU/mL and the corresponding triphenyl formazan yield was 0.072, 0.075, 0.073 and 0.072 μmol for the ethanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. The CFU for the cultures to be shocked with methanol was 0.66×10^8, 0.64×10^8, 0.65×10^8 and 0.64×10^8 CFU/mL and the corresponding triphenyl formazan yield

Table 5.8. Tukey grouping on jadomycin yield data.

Factors	Level	N	Mean % (AU_{526nm})	Grouping
Temperature (°C)	35	60	0.033	A
	40	60	0.191	B
	45	60	0.186	C
	50	60	0.097	D
Exposure (h)	0.5 h	120	0.124	A
	1.0 h	120	0.132	B
Sampling Time (h)	8	24	0.016	A
	16	24	0.033	B
	24	24	0.079	C
	32	24	0.111	D
	40	24	0.135	E
	48	24	0.158	F
	56	24	0.173	G
	64	24	0.185	H
	72	24	0.190	H
	80	24	0.191	H

Groups with the same letter are not significantly different from each other at the 0.05 level.
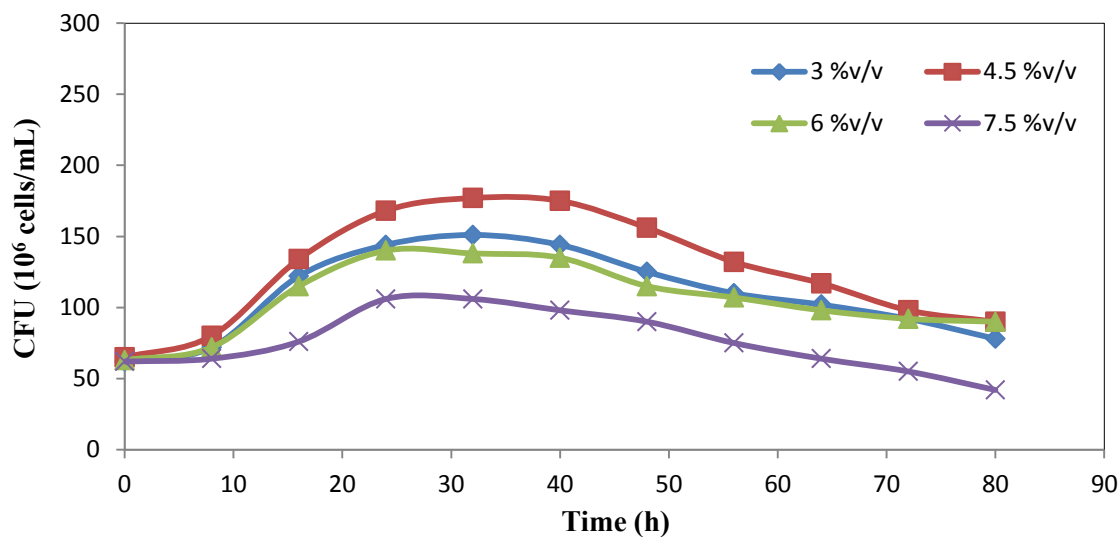
Figure 5.20. Effect of ethanol concentration on the growth of *S.venezuelae* in MSM
production media measured as colony forming units (CFU).
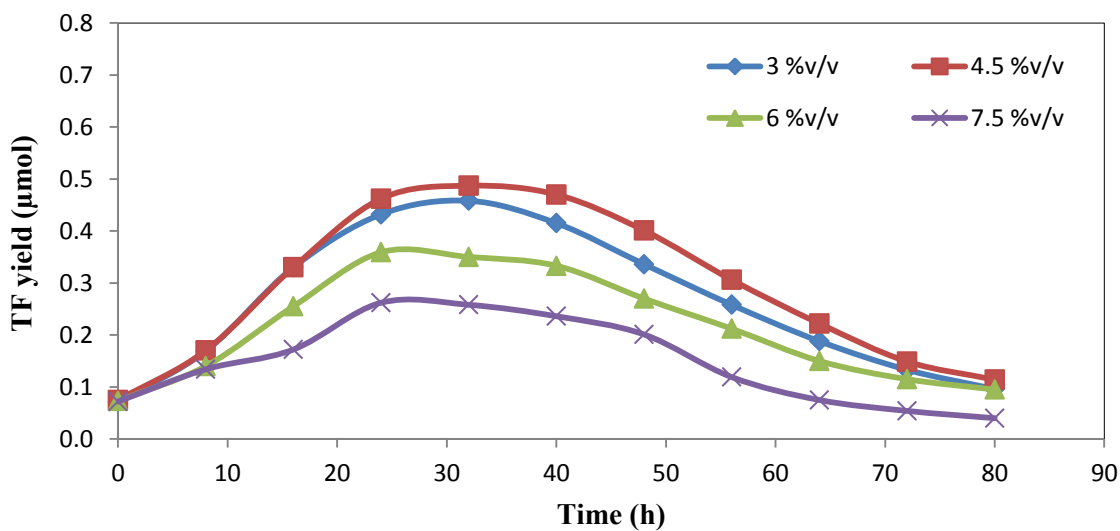
Figure 5.21. Effect of ethanol concentration on the activity of *S.venezuelae* in MSM
production media measured as triphenyl formazan yield (TF).

Figure 5.22. Effect of methanol concentration on the growth of *S.venezuelae* in MSM
production media measured as colony forming units (CFU).

Figure 5.23. Effect of methanol concentration on the activity of *S.venezuelae* in MSM
production media measured as triphenyl formazan yield (TF).

was 0.074, 0.073, 0.075 and 0.072 μmol for the methanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively.

The cell number of the cultures shocked with ethanol increased with time reaching a maximum of 1.56 x 10^8 CFU/mL at the 32 h, 1.80 x 10^8 CFU/mL at the 32 h, 1.40 x 10^8 CFU/mL at the 24 h and 1.06 x 10^8 CFU/mL at the 24 h for the ethanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. The TF yield also increased to a maximum of 0.458 μmol at the 32 h, 0.487 μmol at the 32 h, 0.359 μmol at the 40 h and 0.262 μmol at the 32 h for the ethanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively.

For the culture shocked with methanol, the cell number increased with time reaching a maximum of 2.25 x 10^8 CFU/mL at the 40 h, 2.16 x 10^8 CFU/mL at the 32 h, 1.98 x 10^8 CFU/mL at the 32 h and 1.61 x 10^8 CFU/mL at the 40 h, for the methanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. The TF yield also increased to a maximum of 0.595, 0.545, 0.487 and 0.352 μmol for the methanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively.

The cell number for the cultures shocked with ethanol declined reaching 0.78 x 10^8, 0.90 x 10^8 and 0.90 x 10^8 and 0.42 x 10^8 CFU per mL at the end of the experiment (80 h) for the ethanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. The corresponding final triphenyl formazan (TF) yield was 0.096 μmol, 0.114 μmol, 0.095 μmol and 0.040 μmol for the ethanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively.

For the methanol shocked cultures, the cell number declined reaching 1.31 x 10^8, 1.28 x 10^8, 1.12 x 10^8 and 0.72 x 10^8 CFU per mL at the end of the experiment (80 h) for the methanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. The corresponding final triphenyl formazan (TF) yield was 0.298 μmol, 0.275 μmol, 0.223 μmol and 0.095 μmol for the methanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively.

The results indicated that the highest cell number and highest TF were not reached at the same time for all treatments. The highest cell numbers were produced at the concentrations of 3.0 and 4.5 % v/v for methanol and ethanol, respectively. However, the culture shocked with methanol at a concentration of 3 % v/v resulted in highest cell number and TF yield during the entire period of growth in the MSM production medium.

Analyses of variance (ANOVA) were performed on the TF yield data using Minitab (Quality Plaza, State College, PA, USA) as shown in Tables 5.9. The results showed that the effects of alcohol type, alcohol concentration and sampling time were significant at the 0.001 level. There were significant interactions between the alcohol type, alcohol concentration and the sampling time at the 0.001 level.

The results obtained from the Tukey grouping are shown in Tables 5.10. The two alcohols were significantly different from one another at the 0.05 level. The highest average TF yield of 0.352 μmol was with methanol. The levels of alcohol concentrations were significantly different from one another at the 0.05 level. The highest average TF yield of 0.363 μmol was achieved at the alcohol concentration of 3 % v/v. All the levels of sampling time were significantly different from one another at the 0.05 level. The highest average TF yield of 0.437 μmol was produced at the 32-40 h.

5.4.2. *Streptomyces venezuelae Specific Actvity*

The specific activity results are shown in Figures 5.25 - 5.26. At the time of inoculation in the nutrient deprived amino acid rich MSM production media, the specific activity was $1.16 \times 10^{-9,}$ 1.15×10^{-9}, 1.16×10^{-9} and 1.16×10^{-9} μmol/CFU for the cultures to be shocked with ethanol at the concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. Similarly, the initial specific activity for the cultures to be shocked with methanol was 1.12×10^{-9}, 1.14×10^{-9}, 1.10×10^{-9} and 1.13×10^{-9} μmol/CFU for the methanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively.

After shocking the culture with alcohol, both the cell number and TF yield increased with time. However, the TF yield increased at a faster rate than that of the cell in the first 32 h, resulting in increases in the specific activity. The specific activity reached 3.03×10^{-9}, 2.75×10^{-9}, 2.56×10^{-9} and 2.47×10^{-9} μmol/CFU for the cultures shocked with ethanol at the concentrations of 3.0, 4.5, 6.0 and 7.5 % (v/v), respectively. Similarly, the highest specific activity for the cultures shocked with methanol was 2.64×10^{-9}, 2.52×10^{-9}, 2.46×10^{-9}, 2.19×10^{-9} and μmol/CFU for the concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively.

Table 5.9. Analysis of Variance for TF yield data.

Source	DF	SS	MS	F	P
Total	239	4.781026			
Model					
Alcohol Type	1	0.673100	0.673100	5229.66	0.001
Alcohol Concentration	3	1.218577	0.406192	3155.91	0.001
SamplingTime	9	2.572321	0.285813	2240.63	0.001
Alcohol Type * Sampling Time	9	0.054374	0.006042	46.94	0.001
Alcohol Type * Alcohol Concentration	3	0.099785	0.033261	258.42	0.001
SamplingTime * Alcohol Concentration	27	0.093465	0.003462	23.90	0.001
Alcohol Type* SamplingTime* Alcohol Concentration	27	0.058812	0.002178	16.92	0.001
Error	160	0.020593	0.000129		

DF : Degree of Freedom
SS : Sum of Square
MS : Mean of Square
R^2 : 0.995
CV : 4.73%

Table 5.10. Tukey grouping on TF yield data.

Factors	Level	N	Mean % (µmol)	Grouping
Alcohol Type	Methanol	120	0.352	A
	Ethanol	120	0.246	B
Alcohol Concentration (%v/v)	3.0	60	0.363	A
	4.5	60	0.350	B
	6.0	60	0.300	C
	7.5	60	0.182	D
Sampling Time (h)	8	24	0.178	A
	16	24	0.308	B
	24	24	0.400	C
	32	24	0.437	D
	40	24	0.437	D
	48	24	0.370	E
	56	24	0.291	F
	64	24	0.228	G
	72	24	0.186	H
	80	24	0.153	I

Groups with the same letter are not significantly different from each other at the 0.05 level.
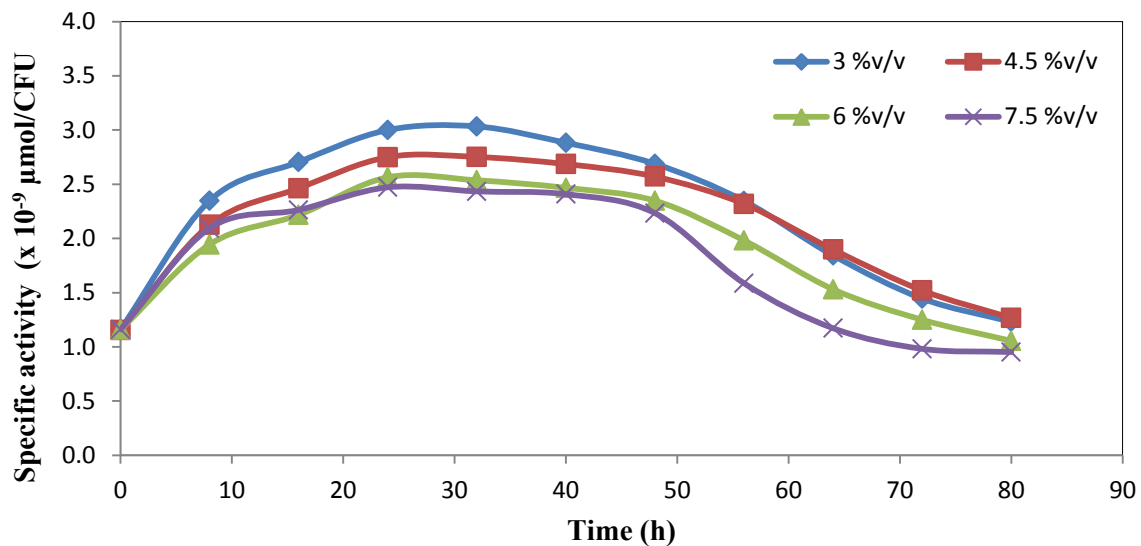
Figure 5.24. The activity of *S.venezuelae* cultures exposed to various ethanol concentrations as measured by specific TF yield in the MSM production media.
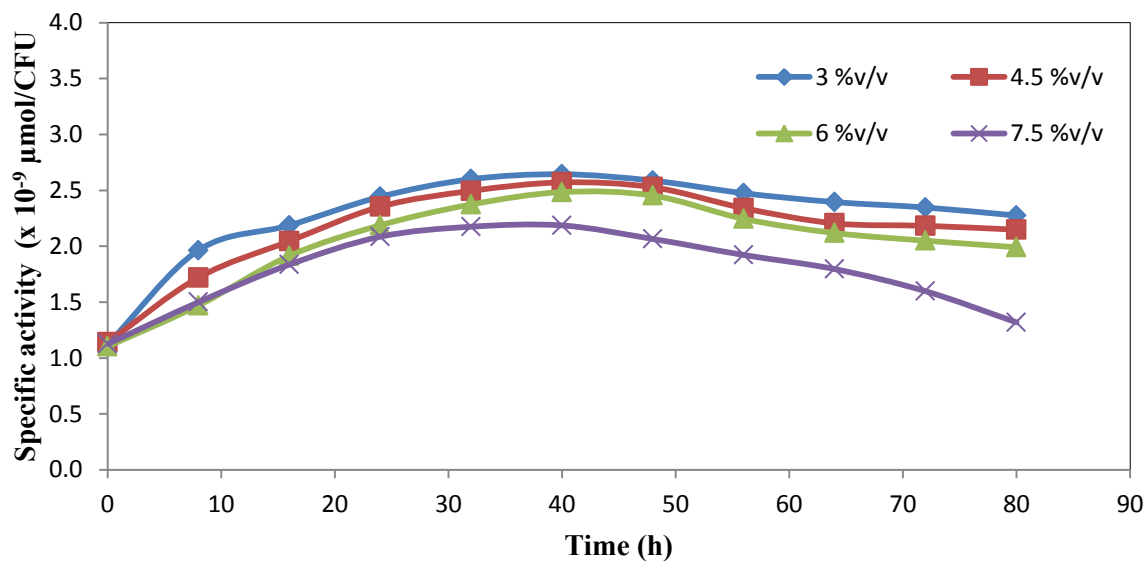
Figure 5.25. The specific activity of *S.venezuelae* cultures exposed to various methanol concentrations as measured by specific TF yield in the MSM production media.

After reaching the maximum growth, the decline in the TF yield was faster than that of the cells and as a result the specific TF slightly declined. The final specific TF was 1.23×10^{-9}, 1.27×10^{-9}, 1.06×10^{-9} and 0.95×10^{-9} μmol/CFU at the end of the experiment (80 h) for the cultures shocked with ethanol at the concentrations of 3.0, 4.5, 6.0 and 7.5 % (v/v), respectively. Similarly, the final specific TF for the cultures shocked with methanol was 2.27×10^{-9}, 2.15×10^{-9}, 1.99×10^{-9} and 1.31×10^{-9} μmol/CFU at 80 h for the methanol concentrations of 3.0, 4.5, 6.0 and 7.5 % (v/v), respectively.

5.4.3. *Jadomycin Production*

Jadomycin B was successfully produced as was indicated by the increase in absorbance (AU 526nm) and the appearance of the dark reddish orange color in the production medium.The jadomycin B production (AU 526) was noted to increase with time after shocking the cultures with ethanol and methanol (Figures 5.27 and 5.28). The final jadomycin amount was 0.475, 0.545, 0.456 and 0.266 AU for the cultures shocked with ethanol at the concentrations of 3.0, 4.5, 6.0 and 7.5 % (v/v), respectively. The final absorbance of jadomycin (80 h) was 0.331, 0.174, 0.435 and 0.412 AU for the cultures shocked with methanol at the concentrations of 3.0, 4.5, 6.0 and 7.5 % (v/v), respectively.

Analyses of variance (ANOVA) were performed on the jadomycin yield data using Minitab (Quality Plaza, State College, PA, USA) as shown in Tables 5.11. The results showed that the effects of alcohol type, alcohol concentration and sampling time on the jadomycin production were significant at the 0.001 level. The results also showed significant interactions between the alcohol type, alcohol concentration and sampling time at the 0.001 level.

The results obtained from the Tukey grouping is shown in Tables 5.12. The two alcohol types were significantly different from one another at the 0.05 level. The highest jadomycin (0.356 AU) was produced with ethanol. The levels of alcohol concentrations were significantly different from one another at the 0.05 level. The alcohol concentration of 6 % (v/v) produced the highest jadomycin (0.435 AU) using methanol and 4.5 % v/v using ethanol (0.558 AU). All the levels of sampling time were significantly different from one another at the 0.05 level. The highest average jadomycin production (0.384 AU) was observed at the 64 h.
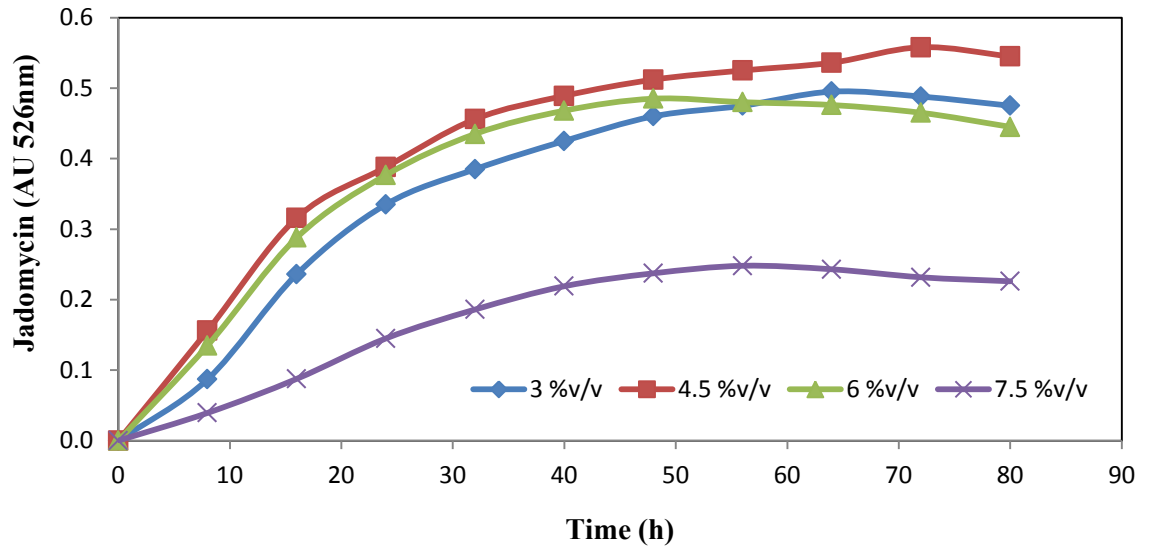
Figure 5.26. Jadomycin production measured as optical density at various ethanol concentrations in the MSM production media.
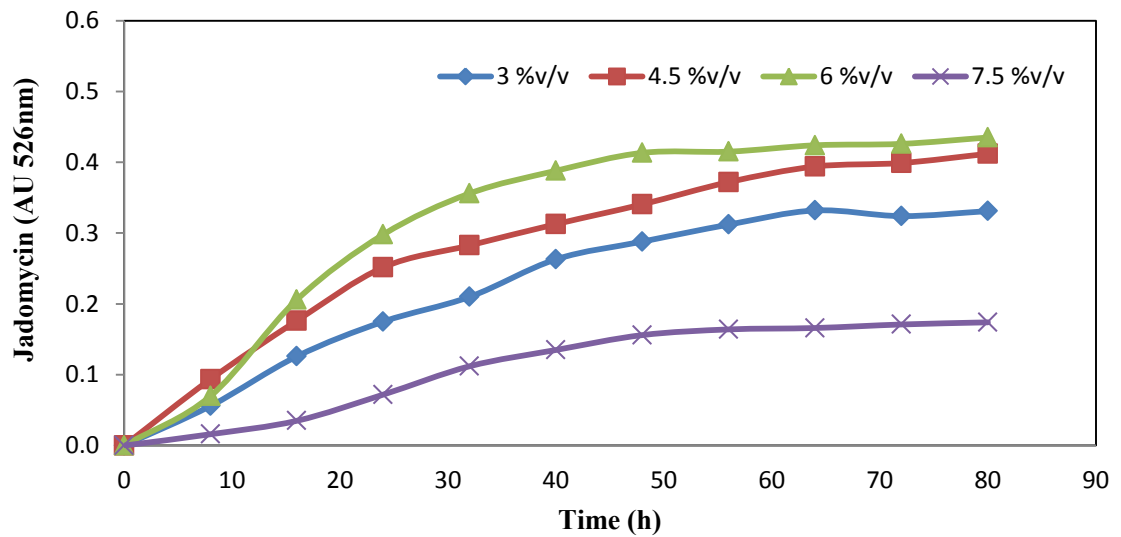
Figure 5.27. Jadomycin production measured as optical density at various methanol concentrations in the MSM production media.

Table 5.11. Analysis of variance for jadomycin production data.

Source	DF	SS	MS	F	P
Total	239	5.178716			
Model					
Alcohol Type	1	0.633454	0.633454	2811.03	0.001
Alcohol Concentration	3	0.549815	0.183272	813.29	0.001
Sampling Time	9	2.262110	0.251346	1115.38	0.001
Alcohol Type * Sampling Time	9	0.030067	0.003341	14.83	0.001
Alcohol Type * Alcohol Concentration	3	1.519895	0.506632	2248.24	0.001
SamplingTime * Alcohol Concentration	27	0.056057	0.002076	9.21	0.001
Alcohol Type * SamplingTime* Alcohol Concentration	27	0.091266	0.03380	15.00	0.001
Error	160	0.036055	0.000224		

DF : Degree of Freedom
SS : Sum of Square
MS : Mean of Square
R^2 : 0.993
CV : 4.82%

Table 5.12. Grouping Information Using Tukey Method for jadomycin production.

Factors	Level	N	Mean% (AU_{526nm})	Grouping
Alcohol Type	Ethanol	120	0.356	A
	Methanol	120	0.253	B
Alcohol Concentration (% v/v)	3.0	60	0.315	A
	4.5	60	0.283	B
	6.0	60	0.375	C
	7.5	60	0.244	D
Sampling Time (h)	8	24	0.081	A
	16	24	0.184	B
	24	24	0.255	C
	32	24	0.305	D
	40	24	0.337	E
	48	24	0.342	F
	56	24	0.375	G
	64	24	0.384	H
	72	24	0.382	H
	80	24	0.380	H

Groups with the same letter are not significantly different from each other at the 0.05 level.

5.5. *Multiple Ethanol Shock in Production Medium*

Experiments were carried out to determine the effect of multiple ethanol (3 % v/v) shocking [once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h)] on the production of jadomycin in MSM production. The colony forming units, dehydrogenase activity (measured through triphenyl formazan (TF) yield) and jadomycin B production were determined at several time intervals. The data are shown in Table E.1 (Appendix E).

5.5.1. *Streptomyces venezuelae Growth*

Figures 5.29 - 5.30 show the effect of multiple ethanol shocpks on the growth of *S.venezuelae* as measured by CFU and TF yield at various time intervals. At the time of inoculations in the nutrient deprived amino acid rich MSM production media, the CFU/mL was 0.56×10^8, 0.58×10^8 and 0.55×10^8 CFU/mL and the corresponding triphenyl formazan yield was 0.063, 0.065 and 0.062 μmol for the cultures to be shocked with 3 % (v/v) ethanol once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively.

The cell number increased with time reaching a maximum of 1.54×10^8 CFU/mL at the 30 h, 1.16×10^8 CFU/mL at the 30 h, and 1.10×10^8 CFU/mL at the 20 h for the cultures shocked with 3 % (v/v) ethanol once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively. The corresponding TF yield also increased to a maximum of 0.463 μmol at the 20 h, 0.256 μmol at the 30 h, and 0.201 μmol at the 30 h for the cultures shocked with 3 % (v/v) ethanol once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively.

The cell number then declined after the first 20 - 30 h reaching 0.78×10^8, 0.36×10^8 and 0.22×10^8 CFU per mL at the end of the experiment (80 h) for the cultures shocked with 3 % (v/v) ethanol once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively. The corresponding final triphenyl formazan (TF) yield was 0.094 μmol, 0.031 μmol and 0.012 μmol for the cultures shocked with 3 % (v/v) ethanol once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively.

The results indicated that the highest cell number and highest TF were not reached at same time for all treatments. However, the culture shocked once (0 h) with 3 % (v/v) ethanol resulted in higher cell number and TF yield during the entire period of growth in the MSM

Figure 5.28. Jadomycin production measured as optical density at various methanol concentrations in the MSM production media.
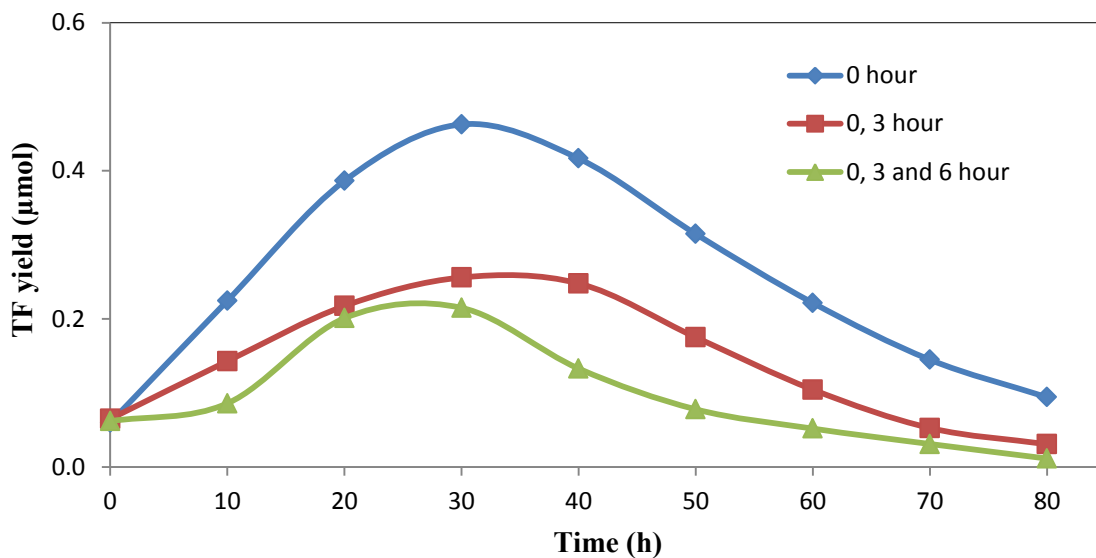
Figure 5.29. Jadomycin production measured as optical density at various methanol concentrations in the MSM production media.

production medium.

Analyses of variance (ANOVA) were performed on the TF yield data using Minitab (Quality Plaza, State College, PA, USA) as shown in Tables 5.13. The results showed that the effects of the number of shocks and sampling time were significant at the 0.001 level. There was a significant interaction between the number of shocks and the sampling time at the 0.001 level.

The results obtained from the Tukey grouping are shown in Tables 5.14. The cultures shocked once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h) were significantly different from one another at the 0.05 level. The highest average TF yield (0.294 µmol) was produced with one shock (0 h). The levels of the sampling time significantly different from each other at the 0.05 level. The highest TF yield (0.314 µmol) was produced at the 30 h.

5.5.2. *Streptomyces venezuelae Specific Activity*

The specific activity (µmol/CFU) was used in this study to assess the level of *S.venezuelae* activity while producing jadomycin in the production medium. The specific activity results are shown in Figure 5.31. At the time of inoculation in the nutrient deprived amino acid rich MSM production media, the specific activity was 1.12×10^{-9}, 1.12×10^{-9} and 1.12×10^{-9} µmol/CFU for the culture shocked with 3 % (v/v) ethanol once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively.

Both the cell number and TF yield increased with time. However, the TF increased at a faster rate than that of the cell number in the first 20 h resulting in increased specific TF. The maximum specific activity was 3.04×10^{-9}, 2.26×10^{-9} and 1.92×10^{-9} µmol/CFU for the culture shocked with ethanol (3% v/v) at once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively. After 20 h, the TF yield declined at a faster rate than that of the cells and as a result the specific activity slightly declined reaching a final value of 1.23×10^{-9}, 0.84×10^{-9} and 0.52×10^{-9} µmol/CFU for the cultures shocked with ethanol (3 % v/v) once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively.

5.5.3. *Jadomycin Production*

Jadomycin B was successfully produced as was indicated by the increase in absorbance

Table 5.13. Analysis of Variance for TF yield data.

Source	DF	SS	MS	F	P
Total	71	1.054180			
Model					
Number of Shocks	2	0.381249	0.190624	338.73	0.001
SamplingTime (h)	7	0.585093	0.083585	148.53	0.001
SamplingTime* Number of Shocks	14	0.060825	0.004345	7.72	0.001
Errors	48	0.027013	0.000563		

DF : Degree of Freedom
SS   : Sum of Square
MS : Mean of Square
R^2 : 0.974
CV : 6.90%

Table 5.14. Turkey's grouping on TF yield data.

Factors	Level	N	Mean % (μmol)	Grouping
Number of Shocks	Once (0 h)	24	0.274	A
	Twice (0, 3h)	24	0.153	B
	Three times (0, 3 & 6 h)	24	0.100	C
Sampling Time (h)	10	9	0.151	A
	20	9	0.268	B
	30	9	0.314	C
	40	9	0.265	D
	50	9	0.167	A
	60	9	0.126	E
	70	9	0.075	F
	80	9	0.045	G

Groups with the same letter are not significantly different from each other at the 0.05 level.
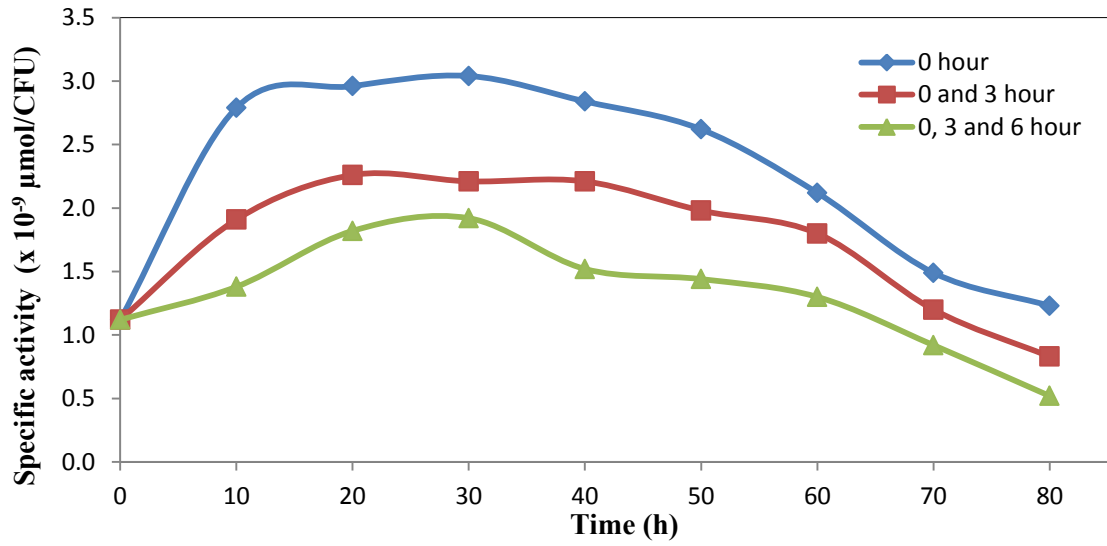
Figure 5.30. The specific activity of *S.venezuelae* cultures shocked with 3% v/v ethanol multiple times as measured by specific TF in the MSM production media.

(AU 526 nm) and the change of color of production media to dark reddish orange. The jadomycin B production [absorbance (AU 526)] was produced immediately after shocking for the culture shocked once (0 h) and after 10 h for cultures shocked twice (0, 3 h) and three times (0, 3 and 6 h) as shown in Figure 5.32. The maximum jadomycin value was 0.492 at 70 h, 0.293 at 60 h and 0.129 at 40 h for the cultures shocked with ethanol (3% v/v) once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively.

Analyses of variance (ANOVA) were performed on the jadomycin yield data using Minitab (Quality Plaza, State College, PA, USA) as shown in Table 5.15. The results showed that the effects of number of shocks and sampling time on jadomycin production were significant at the 0.001 level. The results also showed a significant interaction between the number of shocks and the sampling time at the 0.001 level.

The results obtained from the Tukey grouping are shown in Table 5.16. The ethanol shocks were significantly different from one another at the 0.05 level. The highest jadomycin (0.391 AU) was produced by the culture that was shocked once (0 h). All the levels of sampling times were significantly different from one another at the 0.05 level. The highest jadomycin production (0.312 AU) was produced at the 70 h.

5.6. *Ethanol and Nutrient Shock in Production Medium*

Experiments were carried out to determine the effects of ethanol concentration (3.0, 4.5 and 6.0 % v/v) and nitrogen (L-isoleucine) concentration (45, 60 and 75 mM) on the production of jadomycin in the nutrient deprived amino acid rich MSM production media. The colony forming units, dehydrogenase activity (measured through triphenyl formazan (TF) yield) and jadomycin B production were determined at several time intervals. The data are shown in Tables F.1–F.4 (Appendix F).

5.6.1. *Streptomyces venezuelae Growth*

Figures 5.33 - 5.38 show the effects of various ethanol shocks at different nitrogen concentrations on the growth of *S.venezuelae* measured as CFU and TF yield at several time intervals. At the time of inoculations in the nutrient deprived amino acid rich MSM production media, the CFU for the 3 % (v/v) ethanol shocked cultures was 0.38 x 10^8,
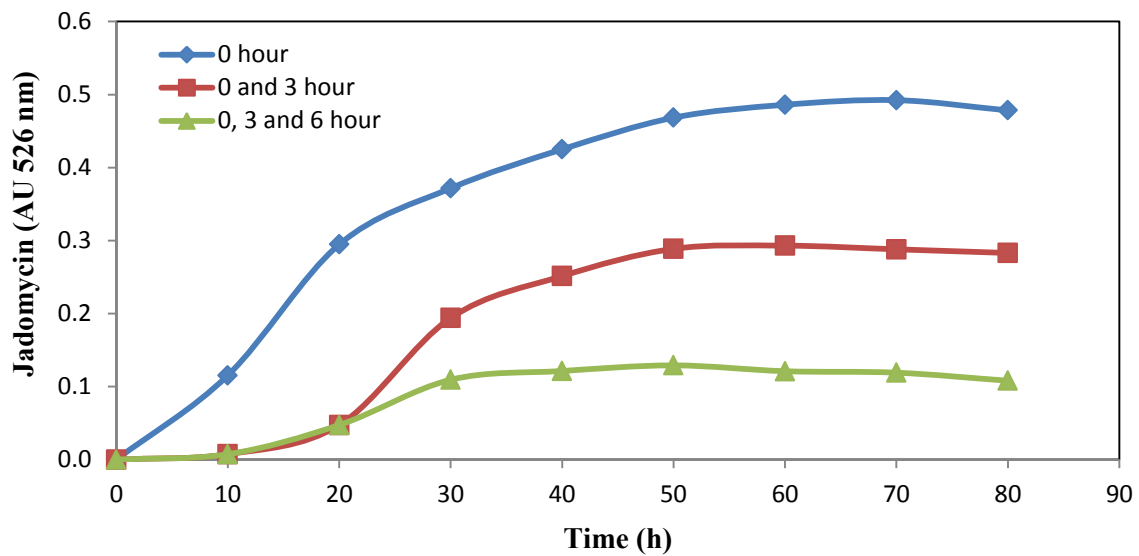
Figure 5.31. Effect of multiple ethanol shocks on jadomycin production in the MSM production media.

Table 5.15. Analysis of Variance for jadomycin production data.

Source	DF	SS	MS	F	P
Total	71	1.761245			
Model					
Sampling Time (h)	7	0.576367	0.082338	624.30	0.001
Number of Shocks	2	1.072089	0.536044	4064.36	0.001
Sampling Time * Number of Shocks	14	0.106458	0.007604	57.66	0.001
Errors	48	0.00633	0.000132		

DF : Degree of Freedom
SS   : Sum of Square
MS : Mean of Square
R^2 : 0.994
CV : 6.88%

Table 5.16. Tukey grouping on jadomycin production data.

Factor	Level	N	Mean % (AU_{526nm})	Grouping
Number of Shocks	Once 0 h	24	0.391	A
	Twice (0, 3) h	24	0.206	B
	Three times (0, 3 & 6) h	24	0.095	C
Sampling Time (h)	10	9	0.040	A
	20	9	0.114	B
	30	9	0.190	C
	40	9	0.232	D
	50	9	0.259	E
	60	9	0.300	F
	70	9	0.312	F
	80	9	0.284	G

Groups with the same letter are not significantly different from each other at the 0.05 level.
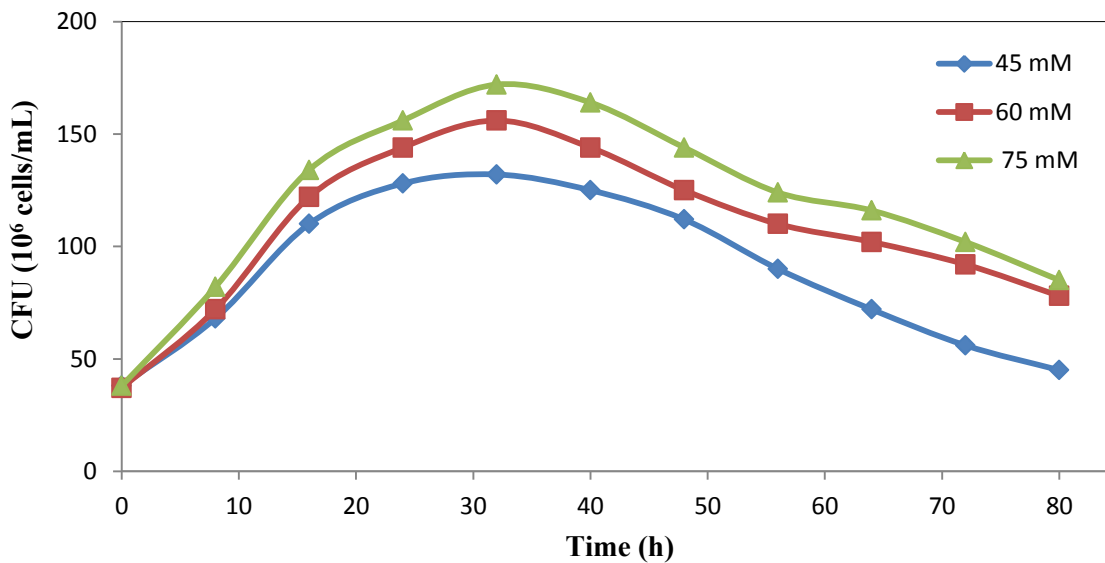
Figure 5.32. Effect of nitrogen concentration on the growth of *S.venezuelae* after being shocked with 3 % v/v ethanol in MSM production media measured as colony forming units (CFU).
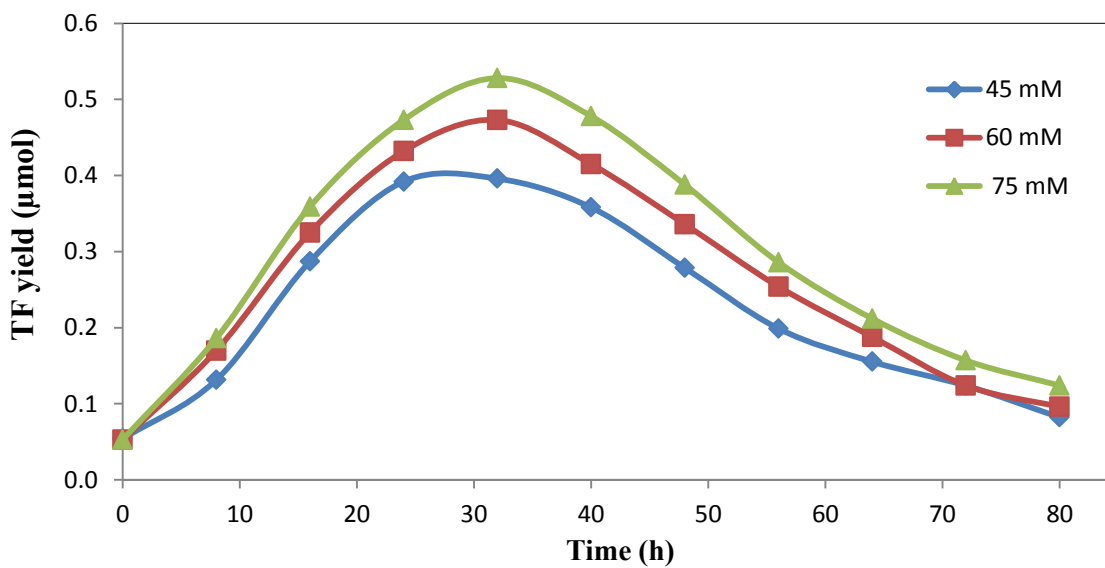
Figure 5.33. Effect of nitrogen concentration on the activity of *S.venezuelae* after being shocked with 3 % v/v ethanol in the MSM production media measured as triphenyl formazan yield (TF).
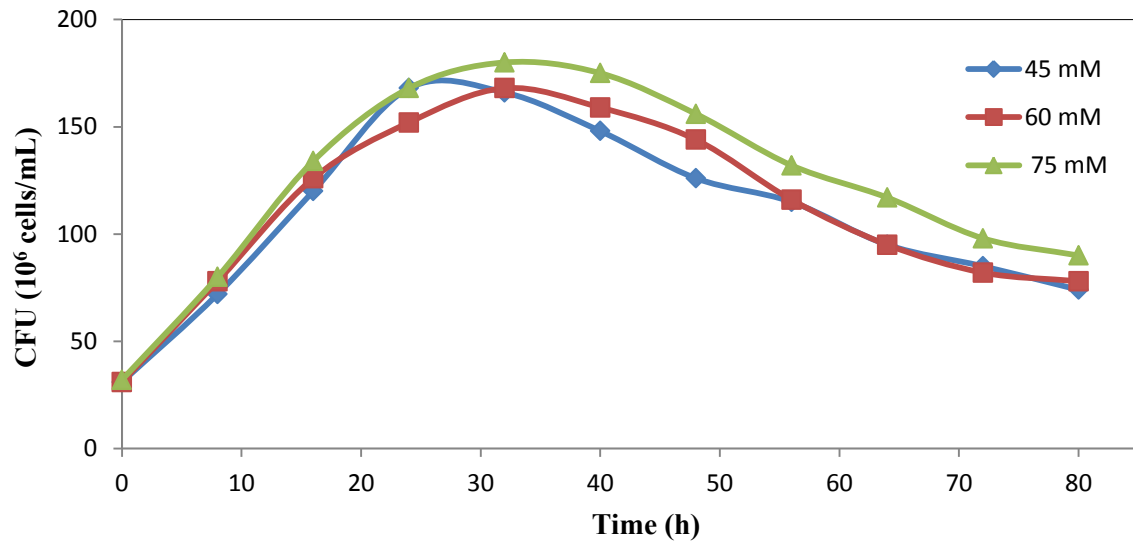
Figure 5.34. Effect of nitrogen concentration on the growth of *S.venezuelae* after being shocked with 4.5 % v/v ethanol in the MSM production media measured as colony forming units (CFU).
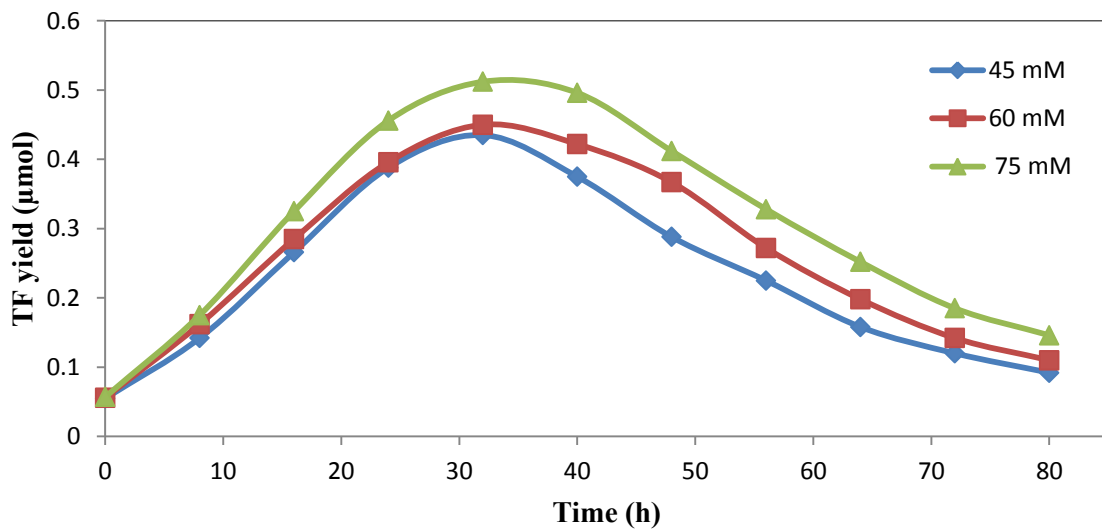
Figure 5.35. Effect of nitrogen concentration on the activity of *S.venezuelae* after being shocked with 4.5 % v/v ethanol in the MSM production media measured as triphenyl formazan yield (TF).
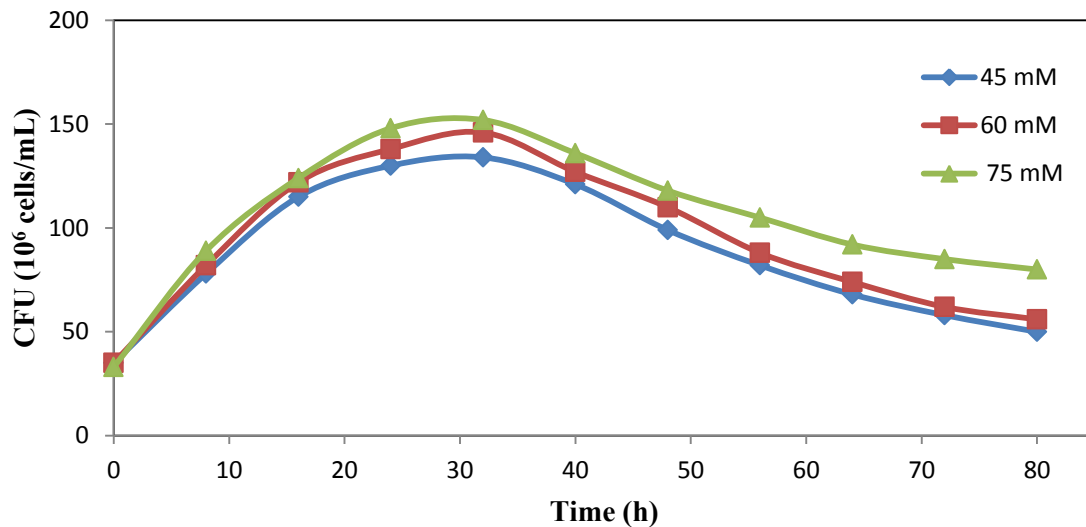
Figure 5.36. Effect of nitrogen concentration on the growth of *S.venezuelae* after being shocked with 6 % v/v ethanol in the MSM production media measured as colony forming units (CFU).
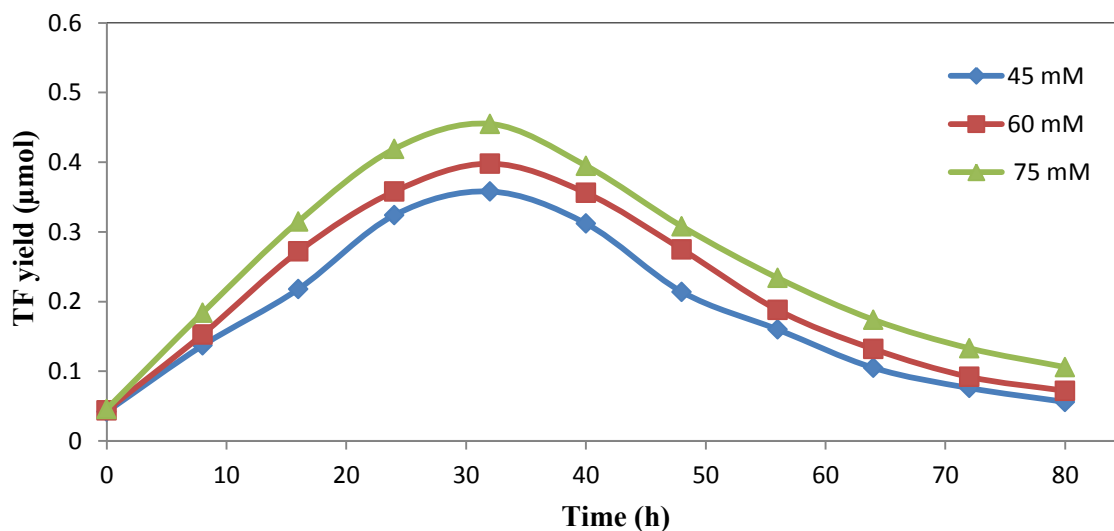
Figure 5.37. Effect of nitrogen concentration on the activity of *S.venezuelae* after being shocked with 6 % v/v ethanol in the MSM production media measured as triphenyl formazan yield (TF).

0.37×10^8 and 0.38×10^8 CFU/mL and the corresponding triphenyl formazan yield was 0.054, 0.053 and 0.053 μmol for the cultures having 45, 60 and 75 mM nitrogen concentrations, respectively. For the culture shocked with 4.5 % (v/v) ethanol, the CFU was 0.31×10^8, 0.31×10^8 and 0.32×10^8 CFU/mL and the corresponding triphenyl formazan yield was 0.044, 0.043 and 0.045 μmol for the culture having 45, 60 and 75 mM nitrogen concentrations, respectively. For the cultures shocked with 6 % ethanol, the CFU was 0.35×10^8, 0.35×10^8 and 0.33×10^8 CFU and the corresponding triphenyl formazan yield was 0.042 μmol, 0.044 μmol and 0.046 μmol for the cultures containing 45, 60 and 75 mM nitrogen, respectively.

For the cultures shocked with 3 % (v/v) ethanol, the cell number increased with time reaching a maximum of 1.32×10^8, 1.56×10^8 and 1.72×10^8 CFU/mL at 32 h for the culture containing 45, 60 and 75 mM nitrogen, respectively. The corresponding TF yield also increased to a maximum of 0.395, 0.473 and 0.528 μmol for the cultures containing 45, 60 and 75 mM nitrogen, respectively. For the cultures shocked with 4.5 % (v/v) ethanol, the cell number increased with time reaching a maximum of 1.68×10^8 CFU/mL at the 24 h, 1.68×10^8 CFU/mL at the 32 h and 1.80×10^8 CFU/mL at the 32 h for the cultures containing 45, 60 and 75 mM nitrogen, respectively. The corresponding TF yield also increased to a maximum of 0.388, 0.450 and 0.512 μmol for the cultures containing 45, 60 and 75 mM nitrogen, respectively. For the cultures shocked with 6 % (v/v) ethanol, the cell number increased reaching a maximum of 1.34×10^8 CFU/mL at the 32 h, 1.46×10^8 CFU/mL at the 32 h and 1.52×10^8 CFU/mL at the 32 h for the cultures containing 45, 60 and 75 mM nitrogen, respectively. The corresponding TF yield also increased to a maximum of 0.358, 0.398 and 0.455 μmol for the cultures containing 45, 60 and 75 mM nitrogen, respectively.

The cell number started to decline after 32 h till the end of the experiment (80 h). The final cell number was 0.45×10^8, 0.78×10^8 and 0.85×10^8 CFU/mL for the cultures shocked with 3 % (v/v) and containing 45, 60 and 75 mM nitrogen, respectively. The corresponding final triphenyl formazan (TF) yield was 0.082, 0.096 and 0.124 μmol for the cultures containing 45, 60 and 75 mM nitrogen, respectively.

For the cultures shocked with 4.5 % (v/v) ethanol, the final cell was reaching 0.74×10^8, 0.78×10^8 and 0.90×10^8 CFU/mL at the end of the experiment (80 h) for the cultures

containing 45, 60 and 75 mM nitrogen, respectively. The corresponding final triphenyl formazan (TF) yield was 0.092 μmol, 0.110 μmol, and 0.146 μmol for the cultures containing 45, 60 and 75 mM nitrogen, respectively. For the cultures shocked with 6 % (v/v) ethanol, the final cell number was 0.50×10^8, 0.56×10^8 and 0.80×10^8 CFU/mL at the end of the experiment (80 h) for the cultures containing 45, 60 and 75 mM nitrgen, respectively. The corresponding final triphenyl formazan (TF) yield was 0.056 μmol, 0.072 μmol, and 0.106 μmol for the culture containing 45, 60 and 75 mM nitrogen, respectively.

The results showed that the culture shocked with 4.5 % (v/v) ethanol and contained 75 mM nitrogen resulted in higher cell number and TF yield during the entire period of growth in the MSM production medium.

Analyses of variance (ANOVA) were performed on the TF yield data using Minitab (Quality Plaza, State College, PA, USA) as shown in Tables 5.17. The results showed that the effects of alcohol concentration, nitrogen concentration and sampling time were significant at the 0.001 level. There were also significant interactions between the alcohol concentration, nitrogen concentration and the sampling time at the 0.001 level.

The results obtained from the Tukey grouping are shown in Tables 5.18. The levels of ethanol concentration were significantly different from one another at the 0.05 level. The highest average TF yield of 0.453 μmol was achieved by the culture shocked with 4.5 % (v/v) ethanol. The levels of nitrogen concentration were significantly different from one another at the 0.05 level. The highest TF yield of 0.302 μmol was achieved by the culture containing 75 mM nitrogen concentration. All the levels of sampling times were significantly different from one another at the 0.05 level. The highest average TF yield of 0.429 μmol was produced at the 32 h.

5.6.2. *Streptomyces venezuelae Specific Activity*

The specific activity results are shown in Figure 5.39. At the time of inoculation in the nutrient deprived amino acid rich MSM production media, the specific activity for the culture shocked with 3 % (v/v) ethanol concentration was 1.42×10^{-9}, 1.43×10^{-9}, and 1.39×10^{-9} μmol/CFU for the cultures containing 45, 60 and 75 mM nitrogen, respectively. For cultures

Table 5.17. Analysis of Variance for TF yield data.

Source	DF	SS	MS	F	P
Total	269	3.955842			
Model					
Nitrogen Concentration	2	0.282826	0.141415	964.46	0.001
Ethanol Concentration	2	0.122471	0.061236	417.63	0.001
Sampling Time	9	0.429801	0.381089	2599.06	0.001
Nitrogen Concentration * Ethanol Concentration	4	0.002972	0.000743	5.07	0.001
Nitrogen Concentration * Sampling Time	18	0.022466	0.001248	8.51	0.001
Ethanol Concentration * Sampling Time	18	0.052760	0.002932	19.99	0.001
Nitrogen Concentration * Sampling Time * Ethanol Concentration	36	0.016149	0.000448	3.06	0.001
Error	180	0.026393	0.000146		

DF : Degree of Freedom
SS : Sum of Square
MS : Mean of Square
R^2 : 0.993
CV : 4.63%

Table 5.18. Grouping Information Using Tukey Method TF yield data.

Factors	Level	N	Mean % (μmol)	Grouping
EthanolConcentration (% v/v)	3.0	90	0.368	A
	4.5	90	0.453	B
	6.0	90	0.388	C
Nitrogen Concentration (mM)	45	90	0.224	A
	60	90	0.275	B
	75	90	0.302	C
Sampling Time (h)	8	27	0.162	A
	16	27	0.294	B
	24	27	0.395	C
	32	27	0.429	D
	40	27	0.404	C
	48	27	0.326	E
	56	27	0.245	F
	64	27	0.182	A
	72	27	0.138	G
	80	27	0.103	H

Groups with the same letter are not significantly different from each other at the 0.05 level.

3.0 % (v/v) ethanol

(a) 4.5 % (v/v) ethanol
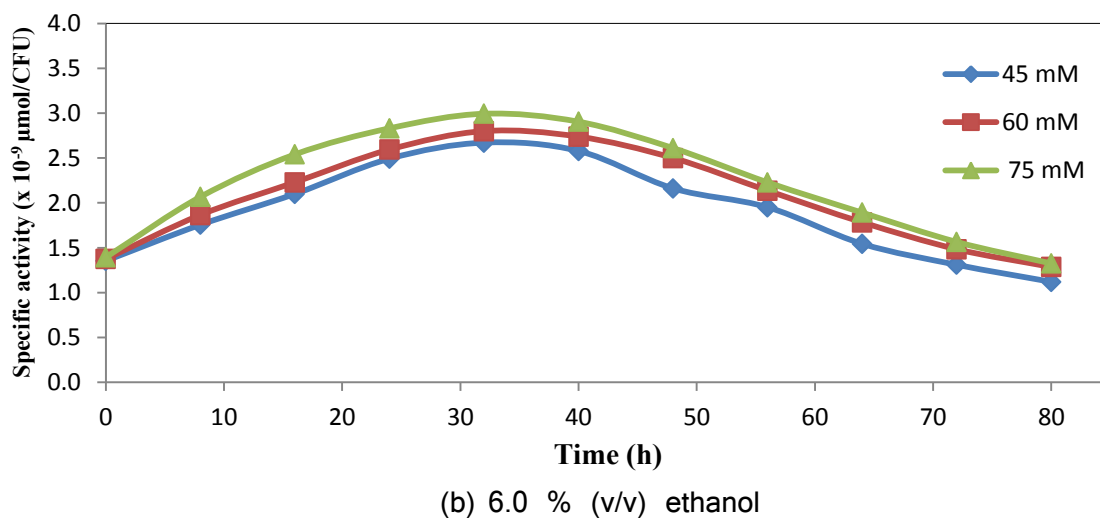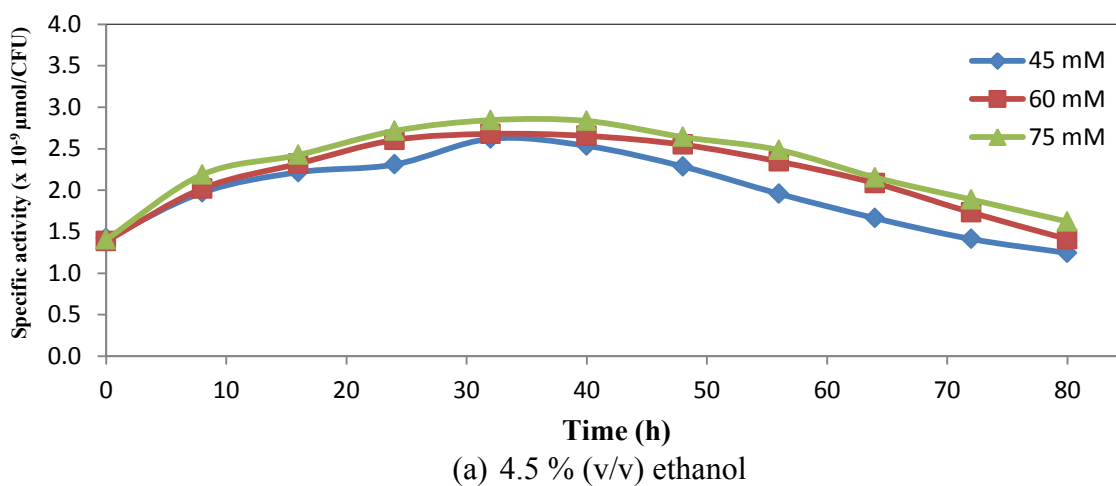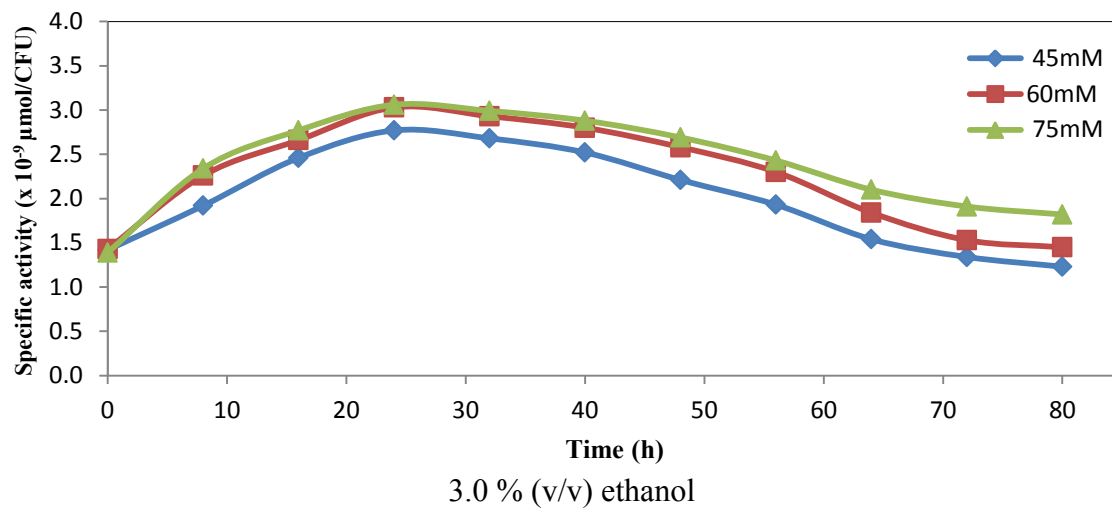
(b) 6.0 % (v/v) ethanol

Figure 5.38. The specific activity of *S.venezuelae* cultures exposed to various ethanol and nitrogen concentrations in the MSM production media as measured by specific TF.

shocked with 4.5 % (v/v) ethanol, the specific activity was 1.41×10^{-9}, 1.38×10^{-9}, and 1.40×10^{-9} μmol/CFU for the cultures containing 45, 60 and 75 mM nitrogen, respectively. For the cultures shocked with 6 % (v/v) ethanol, the specific activity was 1.35×10^{-9}, 1.38×10^{-9}, and 1.39×10^{-9} μmol/CFU for the culture containing 45, 60 and 75 mM nitrogen, respectively.

The TF yield increased at a faster rate than that of the cell during the first 32 h, resulting in increased specific TF. For the cultures shocked with 3 % (v/v) ethanol, the maximum specific activity was 3.01×10^{-9}, 3.03×10^{-9} and 3.06×10^{-9} μmol/CFU at the 32 h for the cultures containing 45, 60 and 75 mM nitrogen, respectively. For the cultures shocked with 4.5 % (v/v) ethanol, the maximum specific activity was 2.30×10^{-9}, 2.67×10^{-9} and 2.84×10^{-9} μmol/CFU at the 40 h for the cultures containing 45, 60 and 75 mM nitrogen, respectively. For the cultures shocked with 6 % (v/v) ethanol, the maximum specific activity was 2.67×10^{-9}, 2.72×10^{-9} and 2.99×10^{-9} μmol/CFU at the 32 h for the cultures containing 45, 60 and 75 mM nitrogen, respectively.

After 32 h, the TF yield declined at faster rate than that of the cells and as a result the specific TF declined. The final specific activity value for the cultures shocked with 3.0 % (v/v) ethanol was 1.23×10^{-9}, 1.45×10^{-9} and 1.82×10^{-9} μmol/CFU for the culture containing 45, 60 and 75 mM nitrogen, respectively. The final specific activity value for the cultures shocked with 4.5 % (v/v) ethanol was 1.24×10^{-9}, 1.41×10^{-9} and 1.62×10^{-9} μmol/CFU for the culture containing 45, 60 and 75 mM nitrogen, respectively. The final specific activity value for the cultures shocked with 6.0 % (v/v) ethanol was 1.12×10^{-9}, 1.28×10^{-9} and 1.32×10^{-9} μmol/CFU for the cultures containing 45, 60 and 75 mM nitrogen, respectively.

5.6.3. *Jadomycin Production*

Jadomycin B was successfully produced as was indicated by the increase in absorbance (Figures 5.40) and the change of color of production medium to dark reddish orange. The jadomycin B production [absorbance (AU 526)] was noted to increase slowly during the first 6 h for the culutres shocked with 3.0 % (v/v) ethanol compared to the cultures shocked with 4.5 and 6.0 % (v/v) ethanol which produced jadomycin immediately after shocking (Figure 5.40).
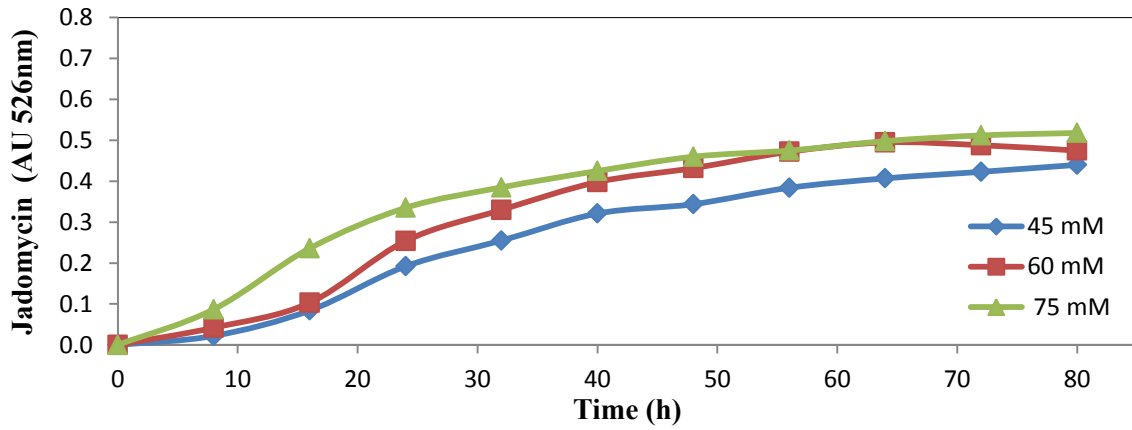
(a) 3.0 % (v/v) ethanol
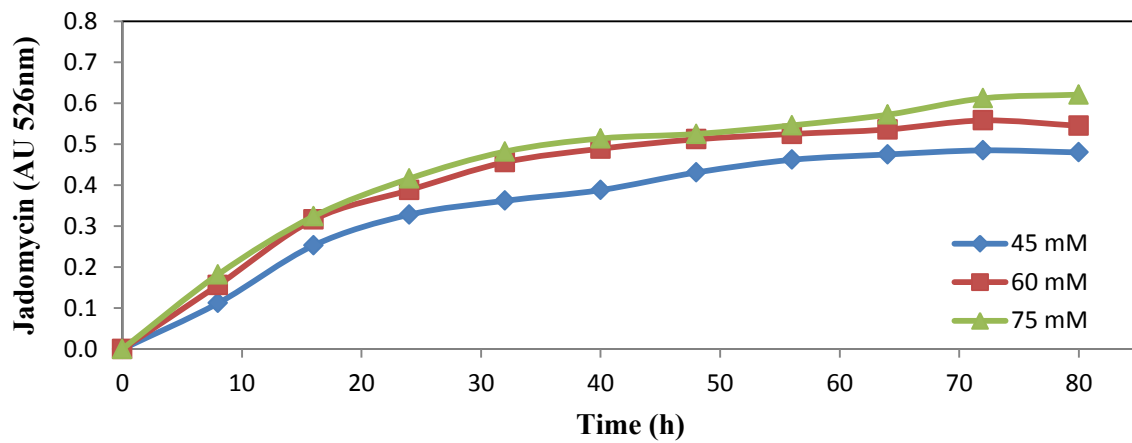
(a) 4.5 % (v/v) ethanol
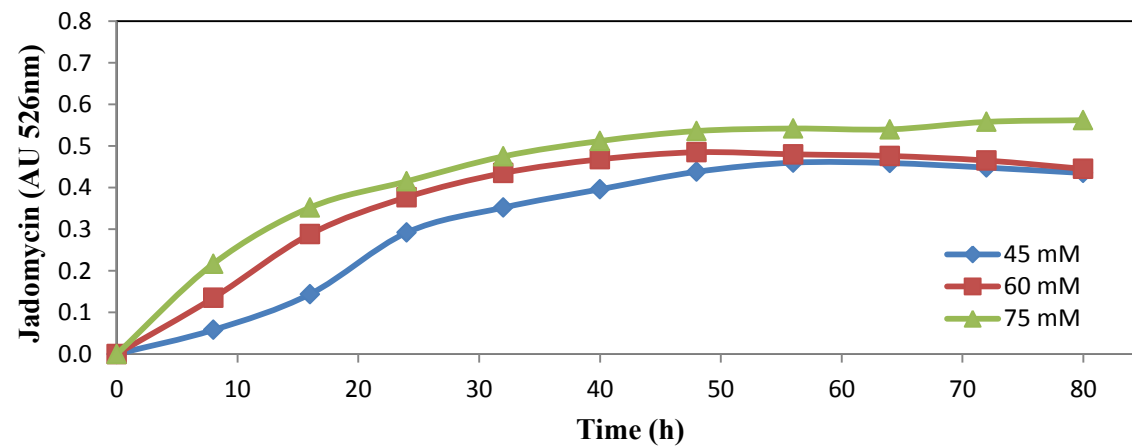
(a) 6.0 % (v/v) ethanol

Figure 5.39. Jadomycin production measured in the MSM production media at various ethanol and nitrogen concentrations.

The final value of jadomycin for cultures shocked with 3.0 % (v/v) ethanol was 0.440, 0.475 and 0.518 AU for the cultures containing 45, 60 and 75 mM nitrogen, respectively. The final value of jadomycin for cultures shocked with 4.5 % (v/v) ethanol was 0.480, 0.545 and 0.621 AU for the cultures containing 45, 60 and 75 mM nitrogen, respectively. The final value of jadomycin for culture shocked at 6.0 % (v/v) ethanol was 0.435, 0.445 and 0.562 AU for the cultures containing 45, 60 and 75 mM nitrogen, respectively.

Analyses of variance (ANOVA) were performed on the jadomycin data using Minitab (Quality Plaza, State College, PA, USA) as shown in Table 5.19. The results showed that the effects of alcohol concentrations, nitrogen concentration and sampling time were significant at the 0.001 level. There were significant interactions between the alcohol concentration, nitrogen concentration and the sampling time at the 0.001 level.

The results obtained from the Tukey grouping are shown in Table 5.20. The levels of ethanol concentration were significantly different from one another at the 0.05 level. The highest average jadomycin of 0.286 AU was achieved by the culture that was shocked with 4.5 % (v/v) ethanol. The levels of nitrogen concentration were significantly different from one another at the 0.05 level. The highest jadomycin (0.429 AU) was achieved with 75 mM nitrogen concentration. All the levels of sampling times were significantly different from one another at the 0.05 level. The highest jadomycin of (0.498 AU) was produced at the 72 h.

Table 5.19. Analysis of Variance for jadomycin production data.

Source	DF	SS	MS	F	P
Total	269	5.4047568			
Model					
Nitrogen Concentration	2	0.188561	0.094281	459.71	0.001
Ethanol Concentration	2	0.351542	0.175771	857.06	0.001
Sampling Time	9	4.24118	0.471569	2299.38	0.001
Nitrogen Concentration * Sampling Time	18	0.056162	0.003120	15.21	0.001
Nitrogen Concentration * Ethanol Concentration	4	0.355208	0.088802	433.00	0.001
Ethanol Concentration * Sampling Time	18	0.046979	0.002610	12.73	0.001
Nitrogen Concentration * Sampling Time * Ethanol Concentration	36	0.125082	0.003475	16.94	0.001
Error	180	0.036915	0.000205		

DF : Degree of Freedom
SS : Sum of Square
MS : Mean of Square
R^2 : 0.994
CV : 3.51%

Table 5.20. Grouping Information Using Tukey Method for jadomycin production

Factors	Level	N	Mean % (AU. $_{526nm}$)	Grouping
Ethanol Concentration (% v/v)	3.0	90	0.238	A
	4.5	90	0.286	B
	6.0	90	0.277	C
Nutrient Concentration (mM)	45	90	0.367	A
	60	90	0.412	B
	75	90	0.429	C
Sampling Time (h)	8	27	0.116	A
	16	27	0.245	B
	24	27	0.338	C
	32	27	0.402	D
	40	27	0.445	E
	48	27	0.476	F
	56	27	0.490	G
	64	27	0.498	H
	72	27	0.504	I
	80	27	0.507	IJ

Groups with the same letter are not significantly different from each other at the 0.05 level.

CHAPTER 6. DISCUSSION

In this study, bench scale fermentation was used to investigate the growth of *Streptomyces venezuelae* and production of jadomycin. Several experiments were carried out in order to develop a standardized method for inoculation and to investigate the effect of different shocking techniques on the induction of jadomycin B production. Shocking was done using heat at different temperatures and exposure times, different alcohol types and concentrations. Multiple shocking with ethanol and nutrient deprivation was also performed to determine the optimum conditions for the jadomycin production by the bacteria.

6.1. *Streptomyces venezuelae Growth*

Jakeman et al. (2006) monitored growth of *S.venezuelae* by measuring the optical density at 600 nm (OD 600). In this study, the growth of *S.venezuelae* population was examined by measuring the optical density at 600 nm (OD 600), the number of colony forming units (CFU) and the triphenyl formazan yield (TF). The result showed that TF yield had a much better correlation with colony forming units (CFU) than that observed between the colony forming units (CFU) and absorbance (OD 600). This was due to the fact that the absorbance measures both live and dead cells while the TF and CFU measure live cells only.

The result showed a lag period of approximately 6 h, during which *S. venezuelae* cells acclimatized to the MYM growth medium and the new environmental conditions and produced the enzyme required for utilization of substrate. Burdock et al. (2011) reported a lag period of approximately 10.3 h for *S.venzuelae* grown in MYM media. Brooks et al. (2012) reported a lag period of 12 h for *S.venezuelae* grown in MYM media. Ghaly et al. (1989) state that after the period of adaptation to new environment, the cells synthesis new enzymes required to utilize the substrate.

After the initial lag period, the *S.venezuelae* grew exponentially reaching 1.98×10^{10} CFU/mL at the 50th h and then declined slightly reaching 1.92×10^{10} CFU/mL at the end of the experiment (65 h). Brooks et al. (2012) reported that *S. venezuelae* had the highest growth (2.25×10^{8} CFU/mL) during the growth phase in MYM broth where the cells were provided with a sufficient amount of nutrients required for sustained growth.

During the exponential growth phase, *S.venezuelae* culture mimics a first-order chemical reaction. The specific growth rate (µ) of the *S.venezuelae* population can then be calculated from the slope of the linear portion of the logarithmic growth curve according to the following equation:

$$\mu = 2.303 \times slope \qquad\qquad (6.1)$$

The specific growth measured in this study was 0.19 h^{-1}. Fattah (2007) reported a maximum specific growth rate of 0.23 h^{-1} for *S.venezuelae* grown in growth media containing soluble starch at 30°C. Glazebrook et al. (1990) reported a maximum specific growth rate of 0.14 h^{-1} for *S.venezuelae* grown in MYM medium at 27°C. Burdock et al. (2011) reported a maximum specific growth rate of 0.23 h^{-1} for *S.venezuelae* grown in MYM medium at 30°C. In the study by MacIntosh (2010), *Streptomyces venezuelae* grown in MYM medium at 32°C had a maximum specific growth rate 0.13 h^{-1}.

The specific TF (µmol/CFU) was calculated by dividing the TF yield by the CFU in order to assess the cell activity during the growth period. The results indicated that the cell activities during the lag phase (3.80 µmol/CFU) and stationary growth phase (4.31 µmol/CFU) were lower than that observed during the exponential growth period (4.41 µmol/CFU). Burdock et al. (2011) found the cell activities of *S.venezuelae* during the lag phase and stationary phase to be lower than that observed during the exponential growth phase. Brooks et al. (2012) found that the specific TF yield remained constant during each of the three growth phases of *Streptomyces venezuelae* indicating the accuracy of TF as a measure of the cell activity. Burdock et al. (2011) also found that the specific TF yield to remain constant during the exponential growth period in MYM broth.

Figures 6.1-6.3 show the growth phases of *S.venezuelae* in the growth media. Figure 6.1 shows the germination of *S.venezuelae* spores in MYM broth medium at 2 h and the vegetative growth at 6 h and Figure 6.2 shows further vegetative growth at 8 and 50 h. Figure 6.3 shows the spore chain formed at 55 h and sporulation at 65 h. During the lag phase, germinating spores were present at 2 h which then appeared as a fairly dense bed of vegetative filamentous hyphae at 6 h. The branching filament is a characteristic feature of

(a) Germinating spore at 2 h
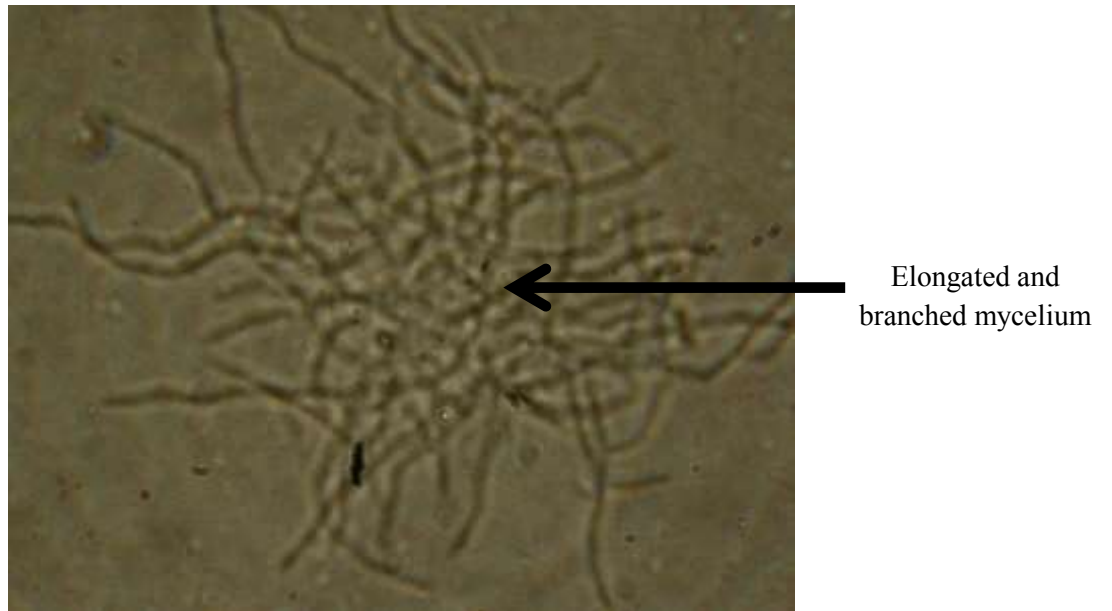
(b) Vegetative growth at 6 h

Figure 6.1. The germination of spore and vegetative growth of *S.venezuelaes* in MYM broth media (Magnification 100 x).
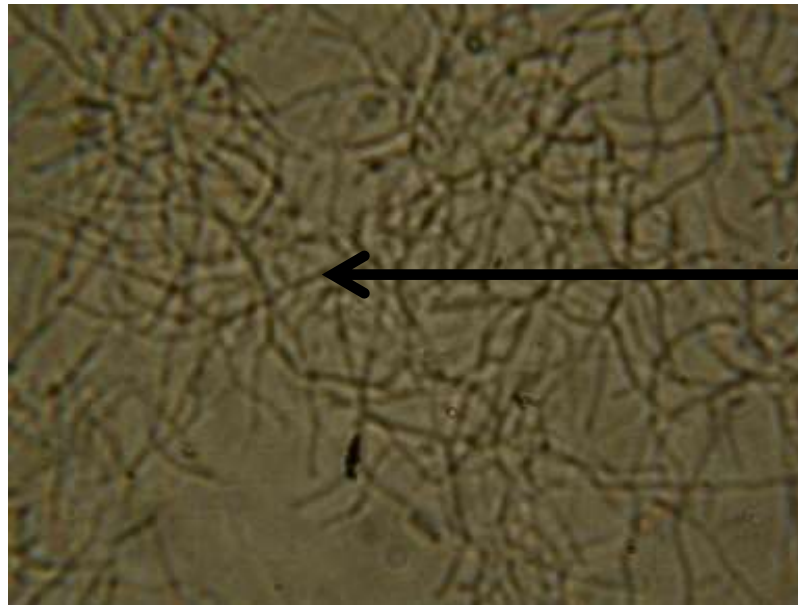
Reduced and branched susbtrate mycelium

(a) Vegetative mycelium at 8 h
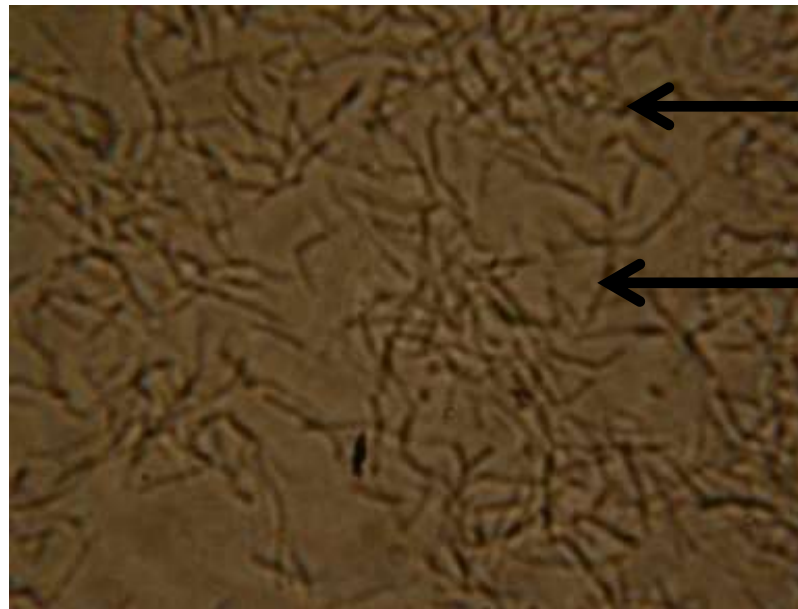
Breakdown of substrate mycelium

Formation of aerial hyphae

(b) Aerial growth at 50 h

Figure 6.2. Growth of *S.venezuelae* in the MYM broth medium during the exponential phase (Magnification 100x).
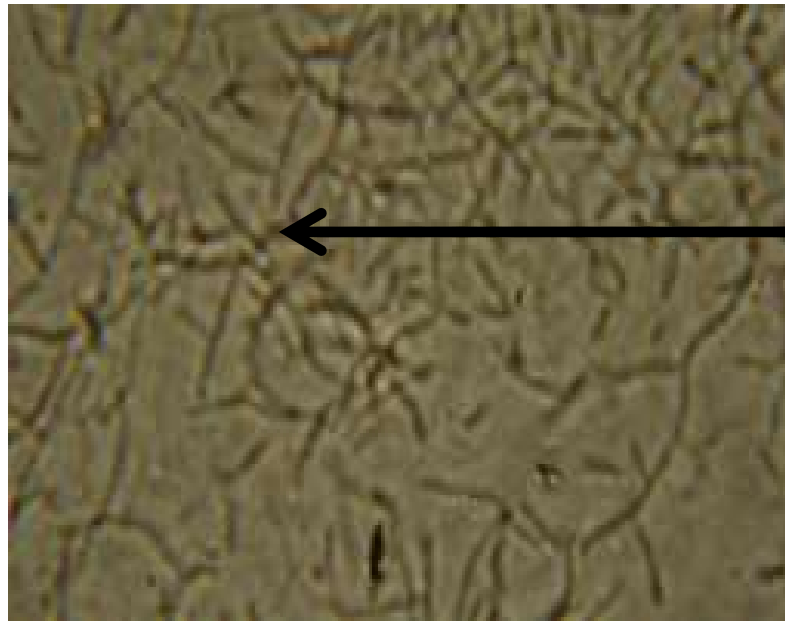
Multiple septation to form unigenomic prespore compartments

(a) Spore chain at 55 h
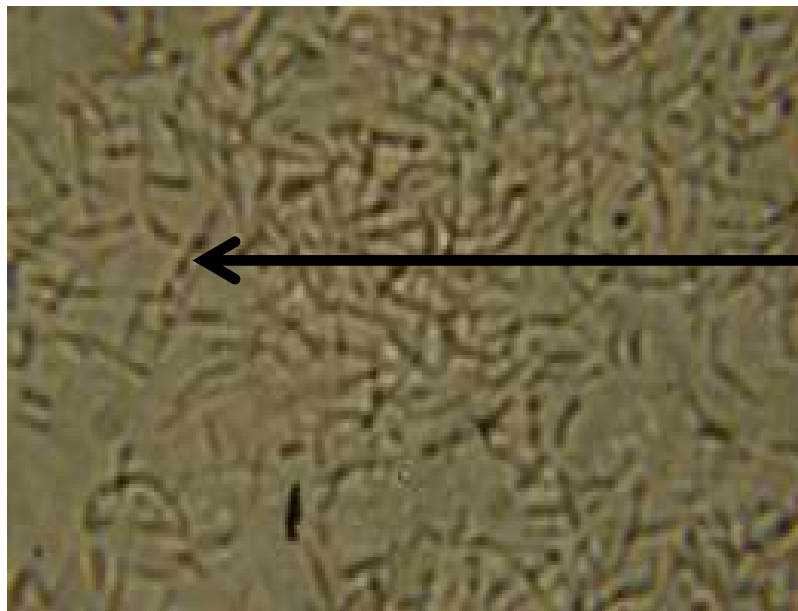
Mature spores in the form of chains

(b) Sporulation at 65 h

Figure 6.3. Growth of *S.venezuelae* in the MYM broth medium during the stationary phase (Magnification 100x).

Streptomyces venezuelae growth. This structure then branched and elongated forming densely uniformed vegetative mycelia (8 h).

The depletion of the nutrient supply in the surrounding environment during the exponential growth period (between 8 - 50 h) resulted in the breakdown of substrate mycelium. The hyphae develop into long, moderately swollen short hyphae, which septated and developed into chains of immature spores that matured and septated after 50 h.

When hyphae extension is stopped (after 50 h), their mutigenomic tips undergo multiple septation to form unigenomic prespore compartments.The samples taken after 55 h (during the stationary growth period) had lower vegetative cells. It is likely that *S.venezuelae* cells experienced nutrient depletion which triggered spore formation. Bradley and Ritz (1968) reported that the first step of sporogenesis in *S.venezuelae* is the simultaneous formation of many septa that allow the hyphae to divide into compartments, which eventually form spores. The author also reported that during stationary and death periods, hyphal ghosts containing membranous vesicles were observed. Glazebrook et al. (1990) reported that the nutritional downshift and stress are often associated with spore formation. Rueda et al. (2001) reported that *S.brasiliens* grown in liquid medium possessed two large compartments separated by a vegetative cross-wall in sporogenic hyphae, where the apical compartment would undergo multiple septation and subsequent development into a chain of spores. Brooks et al. (2012) reported that further disintegration of cells was seen during the stationary phase with the disruption of the outer cell wall and emptying of cytoplasm and scattering of intracellular membrane vesicles throughout the degraded cells.

6.2. *Culture Transfer Time*

Spores of *Streptomyces venezuelae* grown on the soft MYM agar medium were used as an inoculum in order to maintain similar initial physiological state (age and stress) of the initial population. This procedure minimized the inconsistency described in previous studies for jadomycin B production (Jakeman et al., 2006; Doull et al., 1994; Ayer et al., 1991; Rix et al., 2004). A robust and rich culture was developed in a nutrient rich MYM medium. In this study, the effect of *S.venezuelae* culture transfer time (after 18, 21 and 24 h) to nutrient

deprived amino acid rich MSM production medium on cell growth and jadomycin production was investigated.

6.2.1. *Streptomyces venezuelae Growth*

Before the ethanol shock of *S.venezuelae* in the MSM production medium, the initial population size was 0.21×10^8, 0.21×10^8 and 0.20×10^8 CFU/mL for the cultures transferred after 18, 21 and 24 h, respectively. The corresponding TF yield was 0.028, 0.028 and 0.026 µmol for the cultures transferred after 18, 21 and 24 h, respectively.

After administration of ethanol shock in the production medium, the cell number increased reaching a maximum of 1.42×10^8, 1.38×10^8 and 1.36×10^8 CFU/mL after 32 h for the cultures transferred after 18, 21 and 24 h, respectively. The corresponding TF yield was 0.452, 0.433 and 0.412 µmol for the cultures transferred after 18, 21 and 24 h, respectively. While the MSM production media is nutrient-deprived compared to MYM growth media, glucose, nitrogen, and phosphates were still available to the S.venezuelae which caused the cells to divide and the population to increase in cell number during the first 32 h. Holt (1994) reported that the growth of *Streptomyces* from viable fragments of mycelium and the visible remains of cells that have disintegrated indicated the cells were actively growing and the rate of cell growth was greater than the rate of cell death.

The *S.venezuelae* grew exponentially in the MSM production medium during the first 32 h growth phase (from the time of inoculation). The cell growth can be described by the following equation:

$$N_t = No \ e^{\mu t} \tag{6.2}$$

Where:

N_t = the cell number at time t (-)

N_0 = the initial cell number at time t = 0 (-)

μ = the specific growth rate (h^{-1})

t   = time (h)

The specific growth rate (μ) of the *S.venezuelae* population was calculated from the slope of the linear portion of the population growth curve after plotting ln (N_t/N_0) vs time. The specific growth rate (μ) measured in this study was 0.059, 0.059 and 0.057 h^{-1} for the cultures transferred after 18, 21 and 24 h, respectively.

After reaching the maximum growth (at 32 h), the final population size (at 72 h) declined to 1.14×10^8, 1.12×10^8 and 1.04×10^8 CFU/mL for the culture transferred after 18, 21 and 24 h, respectively. The corresponding TF yield (μmol) was 0.155, 0.146 and 0.125 μmol for the cultures transferred after 18, 21 and 24 h, respectively. The cell number declined because of the lack of available nutrients for growth and the synthesis of defensive compounds. The cell decay can be described by the following equation;

$$N_t = N_m \, e^{-kt} \tag{6.3}$$

Where:

N_m = is the maximum cell number (-)

k   = is the cell decay rate (h^{-1})

The specific decay rate (k) of the *S.venezuelae* population was calculated from the slope of the linear portion of the population decay curve after plotting ln (N_t/N_m) vs time. The specific decay rate (k) measured in this study was 0.002, 0.003 and 0.003 h^{-1} for the cultures transferred after 18, 21 and 24 h, respectively.

The images shown in Figures 6.4 - 6.6 and the results presented in Table 6.1 showed that the cells present in the production media at 0 h, 32 h and 80 h post ethanol shock were morphologically different from each other. Brooks et al. (2012) and Wang and Vining (2003) reported that morphological differentiation also occur during the jadomycin production, where the transition of vegetative mycelia to spores are associated with the production phase. In this study, the septation by pinching appeared more common where the septated filaments appeared less turgid and rigid-looking cross walls were observed. The septation and thinning of mycelia are characteristics of *Streptomyces* ageing (Pons et al., (1998)).

Figure 6.4 shows that the vegetative hyphae growth with extensive branching and crossing of hyphae after the culture was transferred to the production medium and before the
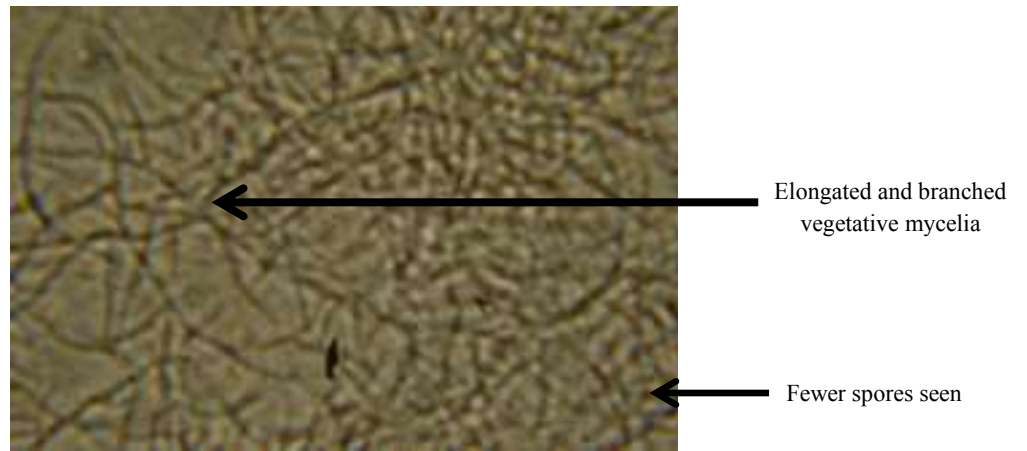
18 h culture
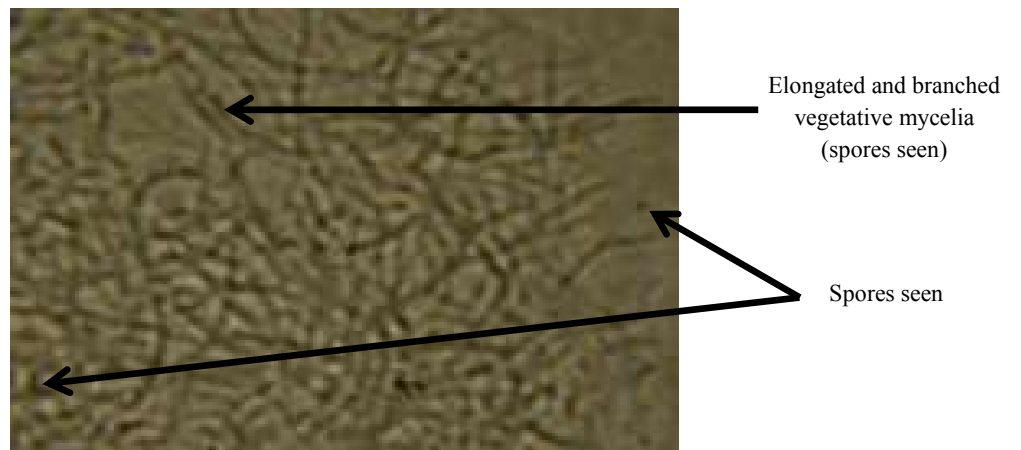
21 h culture
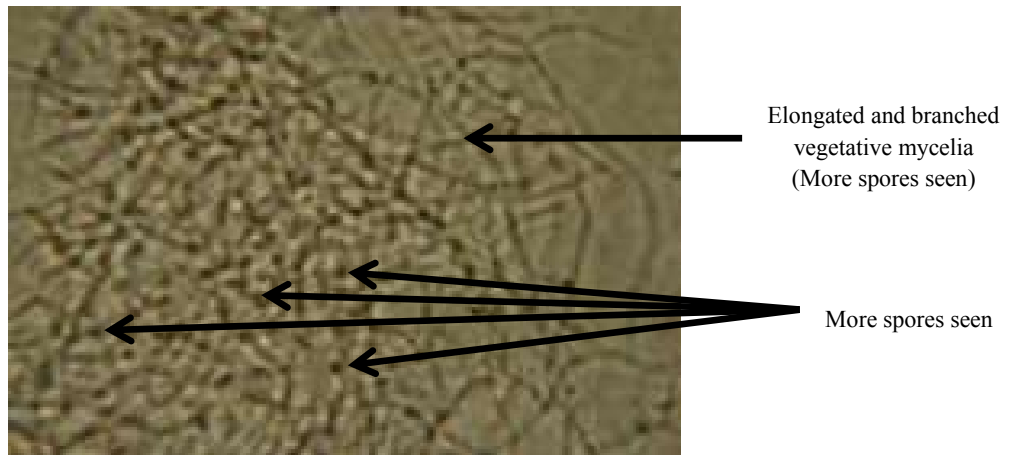
24 h culture

Figure 6.4. Phase contrast microscope image of *S. venezuelae* growth during the time of inoculation (0 h) in MSM production media (Magnification 100x).
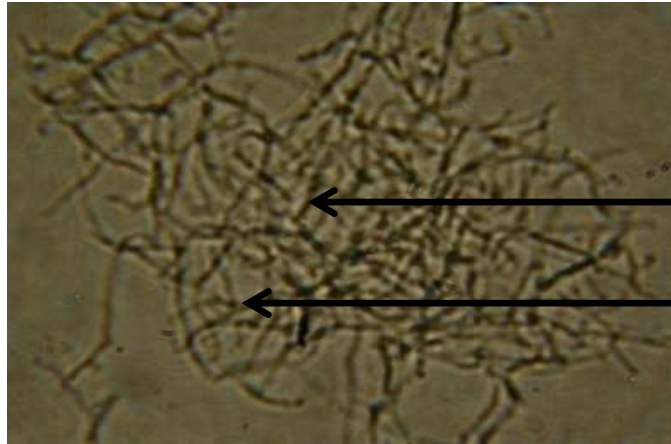
Thick branched aerial hyphae

More thick, branched and cell transition

18 h culture
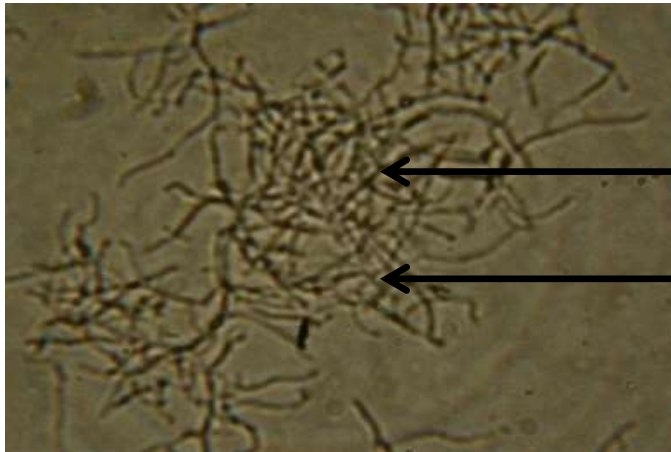
Thick branched aerial hyphae

Less thick, branched and cell transition

21 h culture
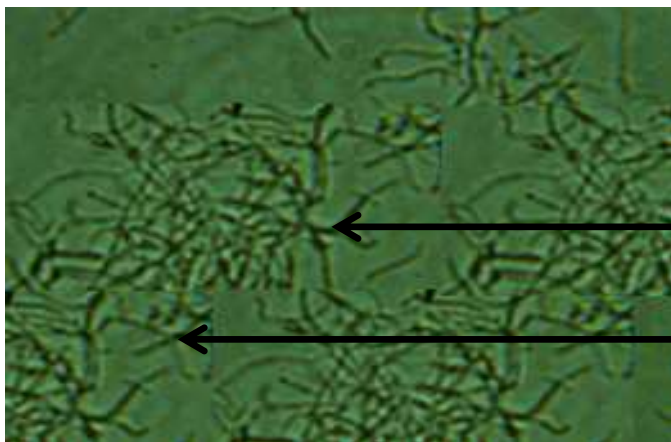
Thick branched aerial hyphae

Loose, unbranched and no cell transition

24 h culture

Figure 6.5. Phase contrast microscope image of *S.venzuelae* growth during maximum growth at 32 h in the MSM production media (Magnification 100x).
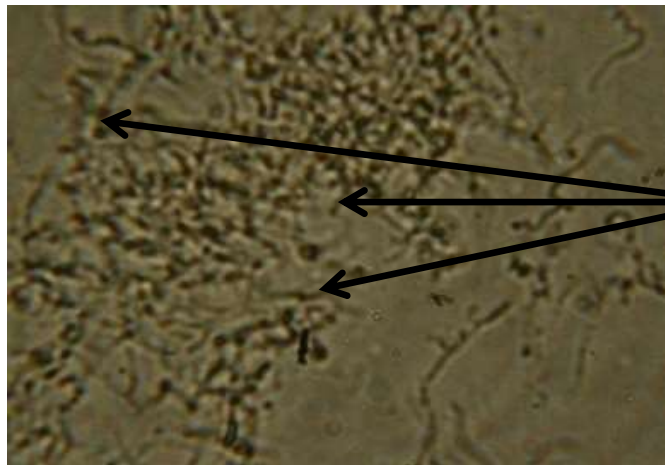
Short spore chain with more aerial hyphae indicating the transition

18 h culture
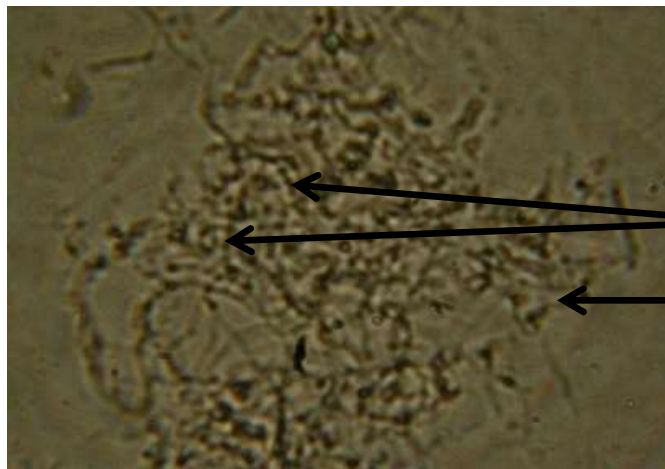
Short spore chain with

Fewer aerial hyphae indicating the transition

21 h culture
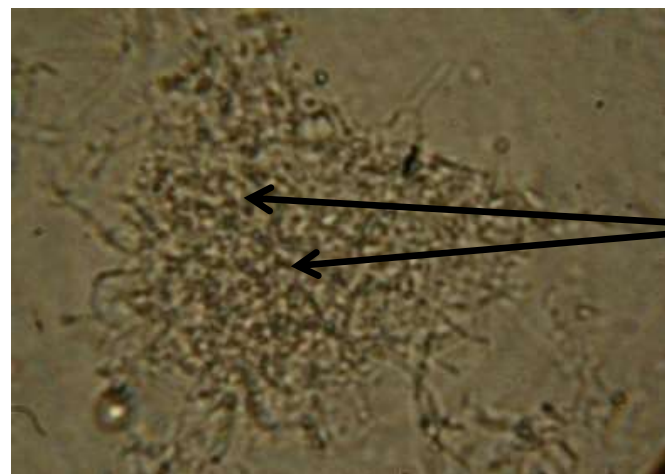
Short spore chain with no aerial hyphae

24 h culture

Figure 6.6. Phase contrast microscope image of *S.venzuelae* growth during final growth at 80 h in the MSM production media (Magnification 100x).

Table 6.1. Changes in growth, activity and jadomycin production of *S.venezuelae* during the various time of culture transfer into MSM production media.

Parameter	Transfer Time (h)		
	18	21	24
Initial (0 h)			
Cells (CFU/mL)	0.21×10^8	0.21×10^8	0.20×10^8
TF yield (µmol)	0.028	0.028	0.026
Specific TF (µmol/CFU)	1.33×10^{-9}	1.33×10^{-9}	1.30×10^{-9}
Maximum (32 h)			
Cells (CFU/mL)	1.42×10^8	1.38×10^8	1.36×10^8
TF yield (µmol)	0.452	0.433	0.412
Specific TF (µmol/CFU)	3.18×10^{-9}	3.13×10^{-9}	3.02×10^{-9}
Final (72 h)			
Cells (CFU/mL)	1.14×10^8	1.12×10^8	1.04×10^8
TF yield (µmol)	0.155	0.146	0.125
Specific TF (µmol/CFU)	1.35×10^{-9}	1.30×10^{-9}	1.21×10^{-9}
Jadomycin (OD_{526nm})	0.430	0.423	0.394

ethanol shock. Brooks et al. (2012) reported that this longitudinal growth of hyphae appears to have little storage material, indicating that the cells are undergoing rapid growth. Bradley and Ritzi (1968) reported that cell wall of a mature spore is much larger (30 mm) in length than the cell wall of vegetative hyphae (15 mm).

Figures 6.5 indicate that cells obtained after 32 h appeared as a bed of filamentous hyphae growth. This is a similar pattern to that reported by Brooks et al. (2012). Their SEM images showed a bed of filamentous hyphal growth with the hyphae appearing as segmented at intervals (0.7 μm) but not uniform in width (varying between 0.3 and 0.5 μm). They also indicated that elongated rod-like cells are the forms of immature spores which have energy reserve vacuoles in smaller portion, as the *S.venezuelae* cells use their energy reserve to actively adjust to the new environment.

The change in elongated branched structure to rigid and turgid looking cell structure in Figure 6.6 indicated that the vegetative cells were undergoing septation and germinating spores in the MSM production media. Further evidence of the transition from vegetative cells to spores was indicated by the incubation time required for the cells to develop on agar plates. Samples taken from inoculation during the first 32 hours after ethanol shock resulted in visible colonies after 24 hours of incubation whereas the CFU development of the samples taken after 32 h required 32 – 40 hours.

Glauert and Hopwood (1961) report that *Streptomyces* spore are surrounded by a thick hyphal wall. Bradley and Ritzi (1968) noted that the *S. venezuelae* cell wall of a mature spore is around 30 nm and appears short whereas the cell wall in vegetative hyphae is typically around 15 nm and appears elongated and branched.. It is possible that the short rod-like cells seen in Figure 6.6 are a form of mature spore. However, it is not known whether this delay in visible colony growth on MYM agar is the result of spore formation or extreme stress.

The decrease in the number of CFUs counted after 32 h could be due to cell death and/or the vegetative cells being transitioned to spores at greater rate compared with that observed during the first 32 h. Multiple septation characteristics of spore formation were completely observed. In this experiment, structural differentiation of *S.venezuelae* was noted during the jadomycin production. The transition from vegetative to spore formation is

associated with this phase where the production rate of jadomycin increased as the spores were formed from the vegetative mycelia during the septation process.

The segmentation initiated by pinching is different from segmentation initiated by cross-walls, as the cross-walls were normally conceived between areas rich in cytoplasmic material where the hyphae appears to be turgid. Bradley and Ritzi (1968) reported that *S. venezuelae* formed sporulating aerial hyphae (comprised of mature spores) on solid media that were held in chains by the presence of a thin outer sheath. Rueda et al. (2001) observed that sporogenic hyphae of *S. brasiliens* cultivated in submerged liquid media were morphologically similar to aerial hyphae growth and developed into spore chain in a comparable manner. Doull et al. (1994) reported that one of the genes involved in jadomycin biosynthesis has also been noted to influence the morphological differentiation. Their result helped to determine whether these conditions could be altered to effect jadomycin production in timely efficient manner.

6.2.2. *Streptomyces venezuelae Specific Activity*

The initial specific activity at the time of inoculation into the MSM production media was 1.33×10^{-9}, 1.33×10^{-9} and 1.30×10^{-9} μmol/CFU for the cultures to be transferred after 18, 21 and 24 h, respectively. The specific activity (μmol/CFU) increased for all transferred cultures and reached its maximum after 32 h post ethanol shock. The maximum specific activity was 3.18×10^{-9}, 3.13×10^{-9} and 3.02×10^{-9} μmol/CFU for the culture transferred after 18, 21 and 24 h, respectively. The increase in specific activity during the first 32 h followed a similar pattern to that of the cell growth. The upward portion of the specific activity curve can be described by the following equation:

$$S_t = S_0 \, e^{at}$$

(6.4)

Where:

S_t = the specific TF at time t (μmol/CFU)

S_0 = the initial specific TF at time t = 0 (μmol/CFU)

a   = the specific TF rate of increase (h^{-1})

The rate of increase in the specific TF (a) was 0.027, 0.025 and 0.011 h^{-1} for the cultures transferred after 18, 21 and 24 h, respectively. The increases in the specific activity indicated that dehydrogenase enzymes within the cells were active. The cells appeared smaller in diameter, uniformed and longer.

After 32 h, the specific activity declined for all cultures. The decrease in the specific activity followed a similar pattern to that of cell decay. The downward portion of the specific activity curve can be described by the following equation:

$$S_t = S_m e^{-dt} \tag{6.5}$$

Where:

S_m = the initial specific activity at time t = 0 (μmol/CFU)

d = the specific activity decline rate (h^{-1})

The specifc activity decline rate (d) was 0.011, 0.011 and 0.012 h^{-1} for the cultures transferred after 18, 21 and 24 h, respectively.

6.2.3. *Jadomycin production*

Jadomycin is only produced when an additional stress (heat or ethanol) is applied to nutritionally unbalanced cultures (Doull et al., 1994; Brooks et al., 2012 and Burdock et al., 2008). Brooks et al. (2012) reported that a gene within the cell is involved in jadomycin biosynthesis and has been found to affect morphological differentiation in *S.venezuelae*. Jakeman et al. (2006) and Rix et al. (2004) reported that *Streptomyces* genuses are able to metabolize the molecules (amino acid) in the production media extracellularly by hydrolytic enzymes.

The jadomycin B concentration increased with time reaching its maximum after 72 h post ethanol shock. The jadomycin production by *S.venezuelae* culture mimics the following first-order chemical reaction:

$$(P_f - P_t) / P_f = e^{-jt}$$
$$(6.6)$$

Where:

P_f = the final maximum jadomycin absorbance (AU 526 nm)

P_t = the jadomycin absorbance measured at time "t" (AU 526 nm)

j = the jadomycin production rate (h^{-1})

The jadomycin production rate (P) was then calculated from the slope of the linear portion of the jadomycin curve after plotting ln ($P_f - P_t$) vs time. The jadomycin production rate (j) measured in this study were 0.074, 0.065 and 0.058 h^{-1} for the cultures transferred after 18, 21 and 24 h, respectively.

The pellet obtained from centrifugation of the samples during obtained from the production media further support the fact that jadomycin B is produced extracellularly by extracellular enzymes from the cells and the uptake of isoleucine and glucose in production media (jadomycin is a highly coloured compound). Several researchers have suggested a possible pathway for jadomycin B production involve the oxidation of the 5, 6-bond on the angucycline intermediate, which then allows for the incorporation of isoleucine (Zheng et al., 2005; Jakeman et al., 2005a: 2006).

6.2.4. *Jadomycin Kinetics*

The jadomycin kinetic parameters are shown in Table 6.2. The results indicated that the culture that was transferred after 18 hour of growth in the nutrient rich MYM growth medium to the nutrient deprived amino acid rich MSM production medium resulted in higher cell number, TF yield, cell activity (specific TF) and jadomycin production. This could be due to the physiological state of the culture as this culture was transferred while the cells were in the exponential growth phase, while the cultures that were transferred after 21 and 24 h were in the process of forming spores.

Daza et al. (1989) suggested that *Streptomyces* (*S. acrimycini*) spores tend to form within 24 h post incubation at 30 ºC. This suggests that the cultures that were transferred after 21 and 24 h might have more spores at the time of transfer into the production medium than the culture that were transferred after 18 h. The transition from vegetative cells to spores was evident during the plate count test as the incubation time required for the cells to develop

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Table 6.2. Jadomycin kinetic parameters a affected by culture transfer time.

Parameters	Culture Transfer Time (h)		
	18	21	24
Growth			
Initial cell number (CFU/mL)	0.21×10^8	0.21×10^8	0.20×10^8
Maximum cell number (CFU/mL)	1.42×10^8	1.38×10^8	1.36×10^8
Cell growth rate (μ) - (0 – 32 h) (h^{-1})	0.059	0.057	0.059
Cell decay rate (k) - (32 – 72 h) (h^{-1})	0.003	0.002	0.003
Activity			
Initial specific activity (μmol/CFU)	1.33×10^{-9}	1.33×10^{-9}	1.30×10^{-9}
Maximum specific activity (μmol/CFU)	3.18×10^{-9}	3.13×10^{-9}	3.02×10^{-9}
Final specific activity (μmol/CFU)	1.35×10^{-9}	1.30×10^{-9}	1.21×10^{-9}
Specific TF increase rate (a) - (0 – 32 h) (μmol/CFU)	0.027	0.025	0.011
Specific TF decline rate (d) - (32 – 72 h) (μmol/CFU)	0.011	0.011	0.012
Jadomycin			
Maximum jadomycin (AU 526nm)	0.430	0.423	0.394
Jadomycin production rate (j) (h^{-1})	0.074	0.065	0.058

on agar plates was much less for the culture that were transferred after 18 h than the cultures that were transferred after 21 and 24 h, respectively.

During the production of secondary metabolites, cells are no longer using nutrients for growth but rather for the synthesis of defensive compounds. The results indicated that the transition from vegetative cells to spores does not occur rapidly but over a period of time. It appears that the dehydrogenase enzymes remained active during the period of jadomycin production, oxidizing organic matter within the cells for the synthesis of defensive compounds.

6.3. *Heat Shock Treatment*

Every bacterial species has an ideal growth temperature that is a characteristic of its physiology (Shuler and Kargi, 1992). *Streptomyces venezuelae* ISP5230 VS1099 is a mesophillic bacteria with an optimum growth temperature of 30ºC. Jadomycin B production is known to be associated with the exposure of *Streptomyces venezuelae* to temperatures higher than 30ºC which indicates the presence of the enzyme responsible for the secondary metabolite biosynthesis within the organisms (Askar et al., 2011).

The aim of this work was to investigate the activity of *S.venezuelae* and production of jadomycin at higher temperatures (35, 40, 45 and 50ºC) with two exposure times (0.5 and 1 hour). These exposure periods were used in this study because studies done on fungal species indicated that heat shocked cultures tend to invoke the production of heat shock proteins soon after shocking (Roychowdhury and Kapoor, 1988). The change in the growth and activity of *S.venezuelae* at different temperatures and exposure times are presented in Table 6.3.

6.3.1. *Streptomyces venezuelae Growth*

Before the heat shock of *S.venezuelae* in the MSM production medium, the initial population size for the cultures to be exposed for 0.5 h was 0.72×10^8, 0.69×10^8, 0.72×10^8 and 0.71×10^8 CFU/mL and the corresponding triphenyl formazan yield was 0.073, 0.071, 0.073 and 0.073 μmol for the cultures to be heat treated at the temperatures of 35, 40, 45 and 50°C, respectively. Similarly, for the cultures to be exposed for 1 h the CFU/mL was 0.70×10^8, 0.71×10^8, 0.70×10^8 and 0.72×10^8 CFU/mL and the corresponding triphenyl

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Table 6.3. Changes in growth, activity and jadomycin production of *S.venezuelae* as affected by heat shock (temperature) and exposure time.

Parameter	Heat Shock Temperature (°C)							
	35°C		40°C		45°C		50°C	
	0.5 h	1 h	0.5 h	1 h	0.5 h	1 h	0.5 h	1 h
Initial								
Cells (CFU/mL)	0.72×10^8	0.70×10^8	0.69×10^8	0.71×10^8	0.72×10^8	0.70×10^8	0.71×10^8	0.72×10^8
TF yield (μmol)	0.073	0.071	0.071	0.072	0.073	0.073	0.073	0.073
Specific TF (μmol/CFU)	1.01×10^{-9}	1.01×10^{-9}	1.02×10^{-9}	1.01×10^{-9}	1.01×10^{-9}	1.04×10^{-9}	1.02×10^{-9}	1.01×10^{-9}
Maximum								
Cells(CFU/mL)	2.04×10^8	1.97×10^8	1.96×10^8	1.82×10^8	1.64×10^8	1.40×10^8	1.33×10^8	1.12×10^8
TF yield (μmol)	0.558	0.524	0.462	0.412	0.356	0.285	0.189	0.131
Specific TF (μmol/CFU)	2.73×10^{-9}	2.65×10^{-9}	2.35×10^{-9}	2.26×10^{-9}	2.17×10^{-9}	2.03×10^{-9}	1.42×10^{-9}	1.16×10^{-9}
Final								
Cells (CFU/mL)	1.76×10^8	1.70×10^8	1.68×10^8	1.52×10^8	1.40×10^8	0.98×10^8	0.84×10^8	0.70×10^8
TF yield (μmol)	0.385	0.374	0.352	0.256	0.134	0.108	0.066	0.055
Specific TF (μmol/CFU)	2.18×10^{-9}	2.20×10^{-9}	2.09×10^{-9}	1.68×10^{-9}	0.95×10^{-9}	1.10×10^{-9}	0.78×10^{-9}	0.78×10^{-9}
Jadomycin (OD$_{526nm}$)	0.042	0.060	0.235	0.268	0.328	0.282	0.155	0.135

formazan yield was 0.071, 0.072, 0.073 and 0.073 μmol for the cultures to be heat treated at the temperatures of 35, 40, 45 and 50°C, respectively.

Heat shock (exposure to high temperatures) response is a cellular reaction that induces the production of whole clusters of proteins called heat shock proteins (HSP). These heat shock protein function as immediate thermal resistance in *S.venezuelae* when exposed to high temperature (Lindquist, 1986). When the bacteria is exposed to higher temperatures (5 - 10ºC higher than the optimal growth temperature of 30°C), these proteins are triggered and the proteins levels are elevated within a short period of time. The heat response is proportional to temperature increase and is short lived (Singh, 1992). Thus, when the culture exposed to temperatures higher than optimal, the HSP concentration reaches a higher level and when the cultures are brought back to normal temperature, these proteins revert back to their pre-shift levels thereby allowing growth to resume (Singh, 1992; Doull et al., 1994).

The cell number after the heat shock increased with time reaching a maximum of 2.04 x 10^8 CFU/mL at 56 h, 1.96 x 10^8 CFU/mL at 56 h, 1.64 x 10^8 CFU/mL at 40 h and 1.33 x 10^8 CFU/mL at the 56 h for the cultures exposed to heat treatment for 0.5 h at the temperatures of 35, 40, 45 and 50°C, respectively. The corresponding TF yield also increased to a maximum of 0.558 μmol, 0.462 μmol, 0.356 μmol and 0.189 μmol for the cultures exposed to heat treatment for 0.5 h at the temperatures of 35, 40, 45 and 50°C, respectively. Similarly, when the cultures were exposed to heat treatment for 1 h, the cell number increased with time to a maximum of of 1.97 x 10^8 CFU/mL at 56 h, 1.82 x 10^8 CFU/mL at 40 h, 1.40 x 10^8 CFU/mL at 32 h and 1.12 x 10^8 CFU/mL at the 32 h for the culture exposed to the temperatures of 35, 40, 45 and 50°C, respectively. The corresponding TF yield also increased to a maximum of 0.524 μmol, 0.412 μmol, 0.285 μmol and 0.131 μmol for the cultures exposed to the temperatures of 35, 40, 45 and 50°C, respectively. The results obtained from this study indicated that the physiological regulation involving the secondary metabolite production by *Streptomyces venezuelae* was triggered by heat shock response. The results also indicated that maximum cell number and TF yield were affected by increases in the temperature and exposure time. The higher the temperature and/or the exposure time the lower are the cell number and cell activity. The highest cell number and TF yield were observed with the exposure temperature of 35°C and the exposure time of 0.5 h.

The growth of *S.venezuelae* post heat exposure at all temperatures (35, 40, 45 and 50°C) and exposure times (0.5 and 1 h) appeared to be exponential. Thus, the cell growth can be described by equation (6.2). The specific growth rate (μ) of the *S.venezuelae* population was calculated from the slope of the linear portion of the growth curve after plotting $\ln (N_t/N_0)$ vs time. The specific growth rate (μ) measured in this study was 0.018, 0.018, 0.019 and 0.011 h^{-1} for the cultures exposed to the heat treatment for 0.5 h at the temperatures of 35, 40, 45 and 50°C, respectively. The specific growth rate (μ) for the culture heat treated for 1 h was 0.018, 0.023, 0.023 and 0.012 h^{-1} at the temperatures of 35, 40, 45 and 50°C, respectively.

After 56 h, the cell number then declined reaching 1.76 x 108, 1.68 x 108, 1.40 x 108 and 0.84 x 108 CFU per mL at the end of the experiment (80 h) for the cultures exposed to heat treatment for 0.5 h at the temperature of 35, 40, 45 and 50°C, respectively. The corresponding final triphenyl formazan (TF) yield for the cultures exposed to heat treatment for 0.5 h at the temperatures of 35, 40, 45 and 50°C was 0.385, 0.352, 0.134 and 0.066 µmol, respectively. Similarly, for the cultures heat treated for 1 h, the cell number declined reaching 1.70×10^8, 1.52×10^8, 0.98×10^8 and 0.70×10^8 at end of the experiment (80 h) for the temperatures of 35, 40, 45 and 50°C, respectively. The corresponding final triphenyl formazan (TF) yield was 0.374, 0.256, 0.108 and 0.055 µmol for the cultures exposed to heat treatment for 1 h at the temperatures of 35, 40, 45 and 50°C, respectively.

The cell decay can be described by the following equation (6.3). The cell decay rate (k) of the *S.venezuelae* population was calculated from the slope of the linear portion of the population decay curve after plotting $\ln (N_t/ N_m)$ vs time. The specific cell decay rate (k) measured in this study was 0.002, 0.002, 0.003 and 0.008 h^{-1} for the cultures exposed to the heat treatment for 0.5 h at temperatures of 35, 40, 45 and 50°C, respectively. The specific cell decay rate (k) for the cultures heat treated for 1 h was 0.002, 0.004, 0.011 and 0.014 h^{-1} at temperatures of 35, 40, 45 and 50°C, respectively.

Although the induction of heat shock proteins (HSPs) is a universal response, a variety of cell activities control synthesis of HSPs in various organisms (Doull et al., 1991). Servant and Mazodier (2001) stated that in *Streptomyces*, the synthesis of vital HSPs (such as DnaK, ClpB, GroEL and HSP18 molecular chaperones) is controlled at the transcriptional level by three different repressors. Inducing the change in temperatures results in synthesizing heat

shock proteins (HSPs) which cause the folding of newly synthesized and denatured proteins and in the transport of other proteins. These proteins are significant for cell growth during the stress conditions, which results in the accumulation of unfolded proteins.

GroEL protein is also essential for growth at all temperatures and initiate fundamental processes such as DNA replication and mRNA transcription in *Streptomyces* (Kumamoto, 1991; Fayet et al., 1989; Wada and Itikawa, 1984). GroEL is associated with specialized metabolic functions including stationary phase metabolism, protein secretion, stringent response and cellular differentiation (Kumamoto, 1991; Martin, 1991; Gomes et al., 1990).

The life cycle of *S.venezuelae* involves the development of basal mycelium from which aerial mycelium is formed and generates spores. The network from the basal mycelium to the aerial mycelial state is indicated by a pause in growth and needed the expression of various genes specific to the aerial mycelium. This morphological differentiation is accompanied by metabolic differentiation. Certain HSPs are known to be involved in controlling the formation of inclusion bodies, in degrading proteins or in mechanisms of secretion (Georgopoulos and Welch 1993; Gottesman et al. 1997).

6.3.2. *Streptomyces venezuelae Specific Activity*

At the time of inoculation of the nutrient deprived amino acid rich MSM production media, the specific activity was 1.01×10^{-9}, 1.02×10^{-9}, 1.01×10^{-9} and 1.02×10^{-9} μmol/CFU for the cultures exposed to the heat treatment for 0.5 h at the temperatures of 35, 40, 45 and 50°C, respectively. Simialrly, the initial specific activity was 1.01×10^{-9}, 1.01×10^{-9}, 1.04×10^{-9} and 1.01×10^{-9} μmol/CFU for the cultures exposed to the heat treatment for 1 h at the temperatures of 35, 40, 45 and 50°C, respectively. The highest specific activity was 2.73×10^{-9}, 2.35×10^{-9}, 2.17×10^{-9} and 1.42×10^{-9} μmol/CFU for the cultures exposed to heat treatment for 0.5 h at the temperatures of 35, 40, 45 and 50°C, respectively. For the cultures exposed to heat shock for 1 h, the maximum specific activity was 2.65×10^{-9}, 2.26×10^{-9}, 2.03×10^{-9} and 1.16×10^{-9} μmol/CFU for the cultures exposed to heat treatment at the temperatures of 35, 40, 45 and 50°C, respectively. The highest specific activity of *S.venezuelae* was observed at the temperature of 35°C, followed by those at the temperatures of 40, 45 and 50°C. The results indicated that the activity was affected by increases in

exposure time and temperature, shorter exposure time and lower temperature favoured *Streptomyces venezuelae* activity.

The increase in specific activity during the growth phase followed similar pattern to that of the cell growth after heat shock. The upward portion of the specific activity curve can be described by equation (6.4). The rate of increase in the specific activity (A) for the culture exposed for 0.5 h was 0.023, 0.020, 0.018 and 0.014 h^{-1} for the temperatures of 35, 40, 45 and 50°C, respectively. For the cultures exposed to heat treatment for 1 h, the specific activity (A) was 0.018, 0.018, 0.024 and 0.041 h^{-1} for the temperatures of 35, 40, 45 and 50°C, respectively. The increases in the specific activity yield indicated that dehydrogenase enzymes within the cells were active and increases in TF yield and cell number were observed during the production of jadomycin B

After reaching the maximum growth, the specific activity slightly declined to 2.18 x 10^{-9}, 2.09 x 10^{-9}, 0.95 x 10^{-9} and 0.78 x 10^{-9} µmol/CFU at the end of the experiment (at 80 h) for the cultures exposed to heat treatment for 0.5 h at the temperature 35, 40, 45 and 50°C, respectively. Similarly, the final specific activity was 2.20 x 10^{-9}, 1.68 x 10^{-9}, 1.10 x 10^{-9} and 0.78 x 10^{-9} µmol/CFU at 80 h for the cultures exposed to heat treatment for 1 h at the temperature 35, 40, 45 and 50°C, respectively.

The downward portion of the specific activity curve can be described by equation (6.5). The specific activity decay rate (D) for 0.5 h exposed cultures was 0.003, 0.002, 0.010 and 0.008 h^{-1}. Whereas, in the 1 h exposed heat shock cultures, the expoenential growth decay (D) was 0.002, 0.005, 0.007 and 0.011 h^{-1}, respectively.

6.3.3. *Jadomycin production*

Jadomycin is only produced when an additional stress is applied to nutritionally unbalanced cultures (Doull et al., 1994; Brooks et al., 2012 and Burdock et al., 2008). Brooks et al. (2012) reported that a gene within the cell is involved in jadomycin biosynthesis and has been found to affect the morphological differentiation in *S.venezuelae*. Jakeman et al. (2006) and Rix et al. (2004) reported those *Streptomyces* genuses are able to metabolize the molecules (amino acid) in the production media extracellularly by hydrolytic enzymes. It is interesting to note that cultures maintained at higher temperatures (35-50°C) than the optimal

growth temperature (30°C) for 0.5 and 1 h have effectively triggered the genes responsible for secondary metabolite production.

The jadomycin B concentration increased with time reaching a maximum after 72 h post heat shock for the cultures exposed for both 0.5 and 1 h, respectively. The jadomycin B concentration increased with time reaching its maximum after 72 h post ethanol shock. Jadomycin production by *S.venezuelae* culture can be described by equation (6.6). The jadomycin production rate (P) was calculated from the slope of the linear portion of the jadomycin curve after plotting ln $(P_f - P_t)$ vs time. The jadomycin production rate (j) measured in this study was 0.008, 0.089, 0.155 and 0.071 h^{-1} for the cultures exposed to heat shock for 0.5 h at the temperature 35, 40, 45 and 50°C, respectively. Similarly, the jadomycin production rate (j) measured for the cultures exposed to heat shock for 1 h was 0.079, 0.118, 0.098 and 0.085 h^{-1} at the temperatures 35, 40, 45 and 50°C, respectively.

Al-Askar et al. (2011) reported that exposure to higher temperature with critical exposure time resulted in increases in the antifungal production and antagonistic activity in *F.solani, F.verticillioides* and *A.alternate*. Sujatha et al. (2005) reported that the best *Streptomyces psammoticus* cell growth and antibiotic production were observed at temperature of 30°C. Shuler and Kargi (1992) reported that incubation temperature directly affects the cell growth and every species has an ideal temperature for growth and antibiotic production that is influenced by its physiology. Al-Askar et al. (2011) reported that *S.spororaveus* RDS28 has optimum temperature of 31°C for its growth and enzyme activity responsible for the secondary metabolite biosynthesis.

Doull et al. (1994) reported that heat treated fermentations containing *S.venezuelae* accumulated maximum jadomycin B titers which peaked 12 hours post exposure and then began to decline at the end. The study also concluded that cultures maintained at 30°C without heat shock accumulated negligible amounts of jadomycin B production, while cultures maintained at 37°C and 42°C for 1 h produced significant amount of jadomycin. This indicates that the genes with in the cell for jadomycin B biosynthesis are being transcribed for less period of time following the initiation of heat stress response.

Guglielmi et al. (1991) reported that an HSP with an unusual size and charge was actively over produced when shifted from 30°C to 41°C upon heat shock thereby revealing its structural and regulatory characteristics. Lindquist and Craig (1988) reported that there is possibility that GroEL proteins play similar roles in the biosynthesis of secondary metabolites. The GroEL proteins may allow the export of insoluble peptide or low-molecular-weight peptide molecules (such as antibiotics) and tend to play a significant role in the multi enzyme complex assembly resulting in secondary metabolite synthesis containing peptide or polyketide bonds.

In this study, cultures grown at higher temperatures resulted in decreasing cell growth compared with 30°C controls but produced significant amount of jadomycin. These secondary metabolites produced in heat shocked cultures follows the similar production pattern as ethanol shock, as most antibiotics tend to accumulate following the end of the growth period in nutrient deprived amino acid rich production media.

6.3.4. *Jadomycin Kinetics*

The jadomycin kinetic parameters are shown in Table 6.4. The results indicated that the culture that was heat shocked at a temperature of 35°C for 1 h resulted in higher cell number, TF yield and cell activity with minimal jadomycin production. On the other hand, the culture that was heat shocked at a temperature of 40°C for 1 h produced the highest jadomycin. The results also showed that the negative effects of exposure time and temperature on the growth, TF yield and activity of *S.venezuelae* were maximum with the exposure time of 0.5 h and the temperature of 35°C. Higher temperature and longer exposure time cause *S.venzuelae* to undergo a reaction resulting in the production of high heat shock proteins which causes the cells to be dormant (or having reduced the cell growth) and when the culture is brought back to 30°C, these proteins revert back to their pre-shift levels thereby causing the cells to resume their growth and activity (Singh, 1992; Doull et al., 1994). The results showed that the higher the temperature and/or the longer the exposure time the longer the time needed for the cells to regrow and the lower the maximum cell number and cell activity.

Table 6.4. Jadomycin kinetic parameters as affected by heat shock temperature.

Parameters	Heat Shock temperature							
	35 ºC		40 ºC		45 ºC		50 ºC	
	0.5 h	1 h	0.5 h	1 h	0.5 h	1 h	0.5 h	1 h
Growth								
Initial cell number (CFU/mL)	0.72×10^8	0.70×10^8	0.69×10^8	0.71×10^8	0.72×10^8	0.70×10^8	0.71×10^8	0.72×10^8
Maximum cell number (CFU/mL)	2.04×10^8	1.97×10^8	1.96×10^8	1.82×10^8	1.64×10^8	1.40×10^8	1.33×10^8	1.12×10^8
Cell growth rate (μ) – (0 – 56 h) (h^{-1})	0.0185	0.0184	0.0186	0.0235	0.0195	0.0239	0.0112	0.0125
Cell decay rate (k) – (56 – 80 h) (h^{-1})	0.0026	0.0026	0.0027	0.0045	0.0039	0.0114	0.0082	0.0146
Activity								
Initial specific activity (μmol/CFU)	1.01×10^{-9}	1.01×10^{-9}	1.02×10^{-9}	1.01×10^{-9}	1.01×10^{-9}	1.04×10^{-9}	1.02×10^{-9}	1.01×10^{-9}
Maximum specific activity (μmol/CFU)	2.73×10^{-9}	2.65×10^{-9}	2.35×10^{-9}	2.26×10^{-9}	2.17×10^{-9}	2.03×10^{-9}	1.42×10^{-9}	1.16×10^{-9}
Specific TF increase rate (a) – (0 – 56 h)	0.023	0.018	0.020	0.018	0.018	0.024	0.014	0.041
Specific TF decline rate (d) – (56 – 80 h)	0.003	0.002	0.002	0.005	0.010	0.007	0.008	0.011
Jadomycin								
Maximum jadomycin (AU 526nm)	0.044	0.061	0.246	0.271	0.331	0.282	0.158	0.135
Jadomycin Production rate (j) (h^{-1})	0.008	0.079	0.089	0.118	0.155	0.098	0.071	0.085

6.4. *Alcohol Shock Treatment*

According to Singh et al (1991), ethanol treatment is more progressive in stimulating the heat shock response in variety of eukaryotic and prokaryotic bacteria. Although the mechanism by which the ethanol facilitates the heat shock response is unknown, Quoronfleh et al (1990) indicated that the bacterial membrane may be the target for ethanol shock and stress protein inductions may be the result by means of repairing damage. In this study, the effects of alcohol type (ethanol and methanol) and alcohol concentration (3.0, 4.5, 6.0 and 7.5 % v/v) on the production of jadomycin B by *S.venezuelae* were evaluated. The timing of the ethanol addition is very critical. Jakeman et al. (2006) reported that allowing the culture to acclimatize prior to administering the ethanol shock shock failed to produce significantly greater amount of secondary metabolites compared to immediatly shocked cultures. In this study, immediate shock (soon after the transfer of healthy vegetative cells) was administered. The changes in the growth and activity of *S.venezuelae* at different concentrations of ethanol and methanol are presented in Table 6.5.

6.4.1. *Streptomyces venezuelae Growth*

At the time of inoculations in the nutrient deprived amino acid rich MSM production media, the CFU/mL for ethanol shocked cultures was 0.62×10^8, 0.65×10^8, 0.63×10^8 and 0.62×10^8 CFU/mL and the corresponding triphenyl formazan yield was 0.072, 0.075, 0.073 and 0.072 μmol for the ethanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. The CFU/mL for methanol shocked cultures was 0.66×10^8, 0.64×10^8, 0.65×10^8 and 0.64×10^8 CFU/mL and the corresponding triphenyl formazan yield was 0.074, 0.073, 0.075 and 0.072 μmol for the methanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively.

The cell number of the cultures shocked with ethanol increased with time reaching a maximum of 1.56×10^8 CFU/mL at the 32 h, 1.80×108 CFU/mL at the 32 h, 1.40×10^8 CFU/mL at the 24 h and 1.06×10^8 CFU/mL at the 24 h for the ethanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. The TF yield also followed the similar trend and increased to a maximum of 0.458 μmol, 0.487 μmol, 0.359 μmol and 0.262 μmol for the

Table 6.5. Changes in growth, activity and jadomycin production of S.venezuelae as affected by alcohol shock (ethanol and methanol) and concentrations.

Parameter	Alcohol Shock							
	Ethanol				Methanol			
	3.0 %	4.5 %	6.0 %	7.5 %	3.0 %	4.5 %	6.0 %	7.5 %
Initial								
Cells (CFU/mL)	0.62×10^8	0.65×10^8	0.63×10^8	0.62×10^8	0.66×10^8	0.64×10^8	0.68×10^8	0.64×10^8
TF yield (μmol)	0.072	0.075	0.073	0.072	0.074	0.073	0.075	0.072
Specific TF (μmol/CFU)	1.16×10^{-9}	1.15×10^{-9}	1.16×10^{-9}	1.16×10^{-9}	1.12×10^{-9}	1.14×10^{-9}	1.10×10^{-9}	1.12×10^{-9}
Maximum								
Cells(CFU/mL)	1.56×10^8	1.80×10^8	1.40×10^8	1.06×10^8	2.25×10^8	2.16×10^8	1.98×10^8	1.61×10^8
TF yield (μmol)	0.458	0.487	0.359	0.262	0.595	0.545	0.487	0.352
Specific TF (μmol/CFU)	3.03×10^{-9}	2.75×10^{-9}	2.56×10^{-9}	2.47×10^{-9}	2.64×10^{-9}	2.52×10^{-9}	2.46×10^{-9}	2.19×10^{-9}
Final								
Cells (CFU/mL)	0.78×10^8	0.90×10^8	0.90×10^8	0.42×10^8	1.31×10^8	1.28×10^8	1.12×10^8	0.72×10^8
TF yield (μmol)	0.096	0.114	0.095	0.040	0.298	0.275	0.223	0.095
Specific TF (μmol/CFU)	1.23×10^{-9}	1.27×10^{-9}	1.05×10^{-9}	0.95×10^{-9}	2.27×10^{-9}	2.15×10^{-9}	1.99×10^{-9}	1.31×10^{-9}
Jadomycin (OD_{526nm})	0.475	0.545	0.458	0.226	0.331	0.174	0.435	0.412

ethanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. Similarly, for the culture shocked with methanol, the cell number increased with time reaching a maximum of 2.25 x 10^8 CFU/mL at the 40 h, 2.16 x 10^8 CFU/mL at the 32 h, 1.98 x 10^8 CFU/mL at the 32 h and 1.61 x 10^8 CFU/mL at the 40 h for the methanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. The TF yield also increased to a maximum of 0.595 μmol, 0.545 μmol, 0.487 μmol and 0.352 μmol for the methanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively.

The *S.venezuelae* grows exponentially post alcohol shock with both alcohols (ethanol and methanol) at all concentrations (3.0, 4.5, 6.0 and 7.5 % v/v). The cell growth can be described by equation (6.2). The specific growth rate (μ) of the *S.venezuelae* population was calculated from the slope of the linear portion of the growth curve after plotting ln (N_t/N_0) vs time. The specific growth rate (μ) measured in this study was 0.034, 0.037, 0.033, 0.022 h-1 for the cultures shocked with ethanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. Simialrly, the specific growth rate (μ) for methanol concentration measured in this study was 0.036, 0.038, 0.035, 0.020 h^{-1} for the cultures shocked with concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively.

The cell number for the cultures shocked with ethanol declined reaching 0.78 x 10^8, 0.90 x 10^8 and 0.90 x 10^8 and 0.42 x 10^8 CFU per mL at the end of the experiment (80 h) for the cultures shocked with ethanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. The corresponding final triphenyl formazan (TF) yield was 0.096 μmol, 0.114 μmol, 0.095 μmol and 0.040 μmol for the ethanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. For the methanol shocked cultures, the cell number declined reaching 1.31 x 10^8, 1.2^8 x 10^8, 1.12 x 10^8 and 0.72 x 10^8 CFU per mL at the end of the experiment (80 h) for the methanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. The corresponding final triphenyl formazan (TF) yield was 0.298 μmol, 0.275 μmol, 0.223 μmol and 0.095 μmol for the methanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. The cell decay curve can be described by equation (6.3). The cell decay rate (k) of the *S.venezuelae* population was then calculated from the slope of the linear portion of the population decay curve after plotting ln (N_t/ N_m) vs time. The specific cell decay rate (k) measured in this study was 0.008, 0.008, 0.005 and 0.011 h^{-1} for the cultures shocked with ethanol concentrations of 3.0,

4.5, 6.0 and 7.5 % v/v, respectively. Simialrly, for methanol shocked cultures the exponenital decay rate (D) measured was 0.006, 0.006, 0.005 and 0.006 h^{-1} for concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively.

Alcohol induction in *S.venezuelae* inhibits the growth and cell activity by altering their different transport systems (such as general amino acid permease system, glucose uptake and biosynthesis mechanism) within the cell (Bisson, 1999; Alexandre and Charpentier, 1998). Alexandre et al. (2001) reported that alcohol inhibits the cell growth (*S.cerevisiae* and other soil bacteria) by directly targeting the plasma membrane ATPase activity, the fluidity of which is modified during the alcohol stress. This results in the permeability to ionic species, especially protons and increases its inflow. This increase in proton inflow results in the rapid disintegration of the electrochemical gradient within the plasma membrane following rapid intracellular acidification. The second effect of alcohol stress is the alteration caused in membrane lipid compositions and represents an adaptive mechanism in plasma membnrane fluidity (Alexandre and Charpentier, 1998; Bisson, 1999).

The distribution of genes in the *S.venezuelae* genome provides interesting information on the molecular mechanisms that allow the *Steptomyces* cell to survive ethanol stress. Alexandre et al. (2001) and Gasch et al. (2000) reported that after exposure to alcohol stress (ethanol) most of the down-regulated genes reflect cell growth arrest which occurs during different stress treatments causing the cell to save energy and adapt to new environmental conditions. These genes are involved in protein biosynthesis (34%), cell growth (4%), RNA metabolism (13%) and cellular biogenesis (3%).

The response of alcohol shock was found to mimic that of the heat shock response. However, methanol was found to be slightly less effective than ethanol in stressing the bacteria. The result also showed that 4.5 % v/v ethanol concentration was favourable for the growth of *S.venezuelae* when compared with other ethanol concentrations. However, more growth was noticed with 3 and 4.5% v/v of methanol shocked cultures. The results also indicated that the highest cell number and highest TF were not reached at the same time for all treatments. However, the culture shocked with methanol at a concentration of 3 % v/v resulted in higher cell number and TF yield during the entire period of growth and cell activity in the MSM production medium.

From a molecular point of view, information concerning ethanol stress is rather inconsistent. No systematic research work is done to examine the molecular process involved in the alcohol stress response. Piper (1995) and Singh (1991) have reported that some genes like HSP genes have been shown to respond to alcohol stress, the pleiotropic results of ethanol indicate that a large number of genes involved in this specific stress response are still to be disclosed. Furthermore, although specific stress response pathways exist for heat and other stress, we still do not know how ethanol stress signalling occurs in *S.venezuelae*.

Singer and Linsquist (1998) reported that it is not clear whether HSP genes play a similar role in both heat and alcohol shock (i.e. preventing aggregation), resulting in a stabilising effect and assisting the refolding of proteins. These are synthesised upon ethanol stress and have been reported to be involved in stabilising membranes and proteins, and inhibiting protein aggregation. The role of HSPs in ethanol stress is still not well understood. It remains to be determined whether they play a similar role as in heat shock (i.e. a stabilising effect, preventing aggregation and assisting the posterior refolding of proteins). Trehalose (which is considered to be a stress protectant) and HSPs are synthesised upon ethanol stress and have been reported to stabilise membranes and proteins and also to suppress protein aggregation (Alexandre et al., 2001).

Ethanol addition to the *S.venezuelae* cells is generally indicated by preferential distribution of ethanol to the hydrophobic environment of lipid bilayers, resulting in a disruption of membrane structure that adversely affects many membrane-associated processes (Rose, 1993; Lloyd et al., 1993; Jones, 1989).

Studies conducted by Serrano (1991), Cartwright et al. (1987) and Coote et al. (1991) indicated that addition of the ethanol and methanol has been shown to prevent uptake of glucose, maltose, ammonium and amino acids as well as causing the potassium, amino acids and other vital nucleotides to leak from the cell wall causing reduced growth and activity. At the plasma membrane, the increased passive proton inflow results in dissipating the electrochemical potential gradient maintained across the bacterial membrane. This greatly affects the significant functions of plasma membrane electrochemical gradient such as the uptake of essential growth components like amino acids and ammonium ions, potassium balance, and the regulation of intracellular pH. Piper (1995) and Mishra (1993) reported that

the addition of ethanol resulted in the decrease in proton motive forces at the plasma membrane or intracellular pH. Coote et al. (1991) reported that same effects are seen in heat stress. Intracellular pH decline may be a major reason for the decreased growth rate in fermentation system, causing a decreased glycolytic flux observed in cells subjected to heat stress (Neves and Francois, 1992) or ethanol exposure (Mishra, 1993).

The activation and inhibition induced by heat being the same as those induced by ethanol stress is not surprising as ethanol tends to interact in a synergetic way to increase the damage caused by heat stress (Piper, 1995). The adverse effects of ethanol are rendered considerably more severe by either increasing the temperature or the alcohol concentration. The presence of ethanol levels above 3% v/v causes thermal death (Van Uden, 1984).

Van Uden (1984) and Jones (1989) reported that ethanol effects appears from a non-specific, lipid-disordering interaction between ethanol and the cell membrane when using different alkanols (methanol and ethanol) of increasing aliphatic chain length. The changes caused by alkanols are related to their lipid solubility. Kirk and Piper (1991) and Van Uden (1984) reported that inducing methanol (less toxic than ethanol) inactivates the cell by 18-20 % in *Saccharomyces cerevisiae* (the heat shock proteins are induced at about 10 % methanol) whereas, ethanol (cytotoxic at lower levels) induces most heat shock proteins.

In this study, while 6.0 % v/v methanol was required to decrease optimal growth for jadomycin production, only 4.5 % v/v ethanol was needed to achieve the same effect. Curran et al. (1994) and Gropper et al. (1993) reported that as alkanol chain length and hydrophobicity increase, smaller concentrations of the alkano1 (ethanol and methanol) are needed to either inhibit bacterial cell growth to achieve heat activation of the HSP sequence, or induce heat shock proteins in the absence of heat. The study concluded that alcohol with greater hydrophobicity are better chemical stress inducers of heat shock proteins in bacteria and increase the capacity to destabilize the hydrophobic interactions that maintain protein conformations in cells. Addition of ethanol causes destabilization of the hydrophobic interactions within native protein structures in *S.venezuelae* and exposes localized hydrophobic regions on the proteins, leading to the association of chaperones with these destabilized proteins. This may be the signal for activation of heat shock genes in bacteria (Piper, 1995).

6.4.2. *Streptomyces venezuelae Specific Activity*

At the time of inoculation in the nutrient deprived amino acid rich MSM production media, the specific activity was 1.16 x 10^{-9}, 1.15 x 10^{-9}, 1.16 x 10^{-9} and 1.16 x 10^{-9} μmol/CFU for the cultures shocked with ethanol at the concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. Similarly, at the time of inoculation in the nutrient deprived amino acid rich MSM production media, the specific activity was 1.12 x 10^{-9}, 1.14 x 10^{-9}, 1.10 x 10^{-9} and 1.13 x 10^{-9} μmol/CFU for the cultures shocked with methanol at the concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively.

The activity of the *S.venezuelae* was noticed to increase greatly for the ethanol shocked cultures than methanol, but declined significantly at the end of the experiment at 80 h, while for the methanol shocked cultures, the increased *S.venezuelae* activity was observed for concentrations 7.5 and 6 % v/v.

For ethanol shocked cultures, the specific activity increased and reached a maximum of 3.03 x 10^{-9} μmol/CFU at the 32 h, 2.75 x 10^{-9} μmol/CFU at the 40 h, 2.56 x 10^{-9} μmol/CFU at the 24 h and 2.47 x 10^{-9} μmol/CFU at the 24 h for the cultures shocked with ethanol concentrations of 3.0, 4.5, 6.0 and 7.5 % (v/v), respectively. Similarly, for the culture shocked with methanol, the maximum specific activity was 2.64 x 10^{-9} μmol/CFU at the 24, 2.52 x 10^{-9} μmol/CFU at the 24, 2.46 x 10^{-9} μmol/CFU at the 24 and 2.19 x 10^{-9} μmol/CFU at the 24 for the methanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively.

The increase in specific activity during the growth phase followed a similar pattern to that of the cell growth after alcohol shock. The upward portion of the specific TF curve can be described by equation (6.4). The rate of increase in the specific TF (A) was 0.028, 0.047, 0.028 and 0.016 h^{-1} for the cultures shocked with ethanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. Similarly, the specifc activity rate of increase (A) measured in this study for the cultures shocked with methanol was 0.031, 0.029, 0.024 and 0.021 h^{-1} for the methanol concentration of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. The increases in the specific activity yield indicated that dehydrogenase enzymes within the cells were active.

After reaching the maximum growth, the specific activity slightly declined to 1.23 x 10^{-9}, 1.27 x 10^{-9}, 1.05 x 10^{-9} and 0.95 x 10^{-9} μmol/CFU at the end of the experiment (80 h) for

the cultures shocked with ethanol. For the culture shocked with methanol, the specific activity slightly declined from the maximum values to 2.27×10^{-9}, 2.15×10^{-9}, 1.99×10^{-9} and 1.31×10^{-9} µmol/CFU at 80 h for the methanol concentrations of 3.0, 4.5, 6.0 and 7.5 % (v/v), respectively. The downward portion of the specific activity curve can be described by equation (6.5). The specific activity decay rate (D) was 0.010, 0.009, 0.012 and 0.011 h^{-1} for the cultures shocked with ethanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. Simialrly, specifc activity decay rate (D) was 0.001, 0.002, 0.003 and 0.016 h^{-1} for the cultures shocked with methanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively.

6.4.3. *Jadomycin Production*

The antibiotic production by *S.venezuelae* was promoted by exposure to the alcohol shock which was stressed using various concentrations of both ethanol and methanol. Although the process whereby alcohol shock treatment induces the production of jadomycin B has not been determined, Doull et al. (1994) reported on the ability of alcohol treatment to trigger a heat-shock response and effectively act as a source of polyketide precursors or to significantly improve the membrane permeability and thus promote release of the secondary metabolites in *S.venezuelae*. No reports in the literature were found to give clear relation between the antibiotic production and alcohol induced shock in *S.venezuelae*.

The result obtained from this study showed that addition of alcohol at higher concentration than the 3 % v/v reported by Doull et al. (1994) and Jakeman et al (2006) proved to be more effective in inducing the jadomycin production for both alcohol types. However, increasing the concentration above 6 % v/v also affected the growth and secondary metabolite production in the same fashion as high temperature shock.

Ethanol causes bacteria to produce a set of stress proteins, called heat shock proteins (HSP) (Neidhardt and Van Bogelen, 1987). The mechanism by which the ethanol induces jadomycin production is by denaturing proteins (mimicking a heat stress). Singh et al. (1991) suggested that ethanol tend to provide the polyketide precursors by changing the proteins to acetaldehydes. Other possibilities are that ethanol causes stress on the cell membrane of *S.venezuelae* making it more permeable and favoring the export of antibiotic. This shows that ethanol might have actively playing a role as both inducer and precursor supplier for

secondary metabolite production (Doull et al., 1994, Singh, 1991). These similar effects might have also been noticed in methanol induced cultures, but the percentage of inducing these effects might be lesser than ethanol, which caused more cell growth and dehydrogenase activity. However, increases in methanol concentration exhibited a similar effect as ethanol.

The jadomycin B concentration increased with time reaching its maximum after 72 h post ethanol shock. Jadomycin production by *S.venezuelae* culture can be described by equation (6.6). The jadomycin production rate (P) was calculated from the slope of the linear portion of the jadomycin curve after plotting ln $(P_f - P_t)$ vs time. The jadomycin production rate (j) measured in this study was 0.071, 0.054, 0.115 and 0.079 h^{-1} for the cultures shocked with ethanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively. Similarly, the jadomycin production rate (j) measured was 0.077, 0.085, 0.053 and 0.071 h^{-1} for the cultures shocked with methanol concentrations of 3.0, 4.5, 6.0 and 7.5 % v/v, respectively.

Both the alcohols (ethanol and methanol) proved to be effective in inducing jadomycin B production. The effective concentration in this study was 6 % v/v for ethanol and methanol. However, the 4.5 % v/v ethanol concentration exhibited a similar effect to the 6 % v/v ethanol concentration and will be more suitable for shocking the *S.venezuelae* for jadomycin production. *S.venezuelae* was found to be actively growing in the production media despite the alcohol shock and the bacteria were able to utilize the available carbon, nitrogen and phosphates sources for growth and jadomycin production. The 4.5 % v/v alcohol concentration is higher than the 3% v/v reported in previous studies (Doull et al., 1994; Jakeman et al., 2006; Burdock et al., 2008; Brooks et al., 2012) for effective jadomycin production. However, the jadomycin produced in this study was higher.

6.4.4. *Jadomycin Kinetics*

The jadomycin kinetic parameters are shown in Table 6.6. The culture shocked with 3.0 % v/v methanol resulted in higher cell growth, TF yield and cell activity. The increase in concentration of both ethanol and methanol causes inhibition in growth and activity of *S.venezuelae* but increased jadomycin production. The 6 % v/v ethanol concentration resulted

Table 6.6. Jadomycin kinetic parameters as affected by alcohol shock type and concentrations.

Parameters	Ethanol				Methanol			
	3.0 %	4.5 %	6.0 %	7.5 %	3.0 %	4.5 %	6.0 %	7.5 %
Growth								
Initial cell number (CFU/mL)	0.62×10^8	0.65×10^8	0.63×10^8	0.62×10^8	0.66×10^8	0.64×10^8	0.68×10^8	0.64×10^8
Maximum cell number (CFU/mL)	1.56×10^8	1.80×10^8	1.40×10^8	1.06×10^8	2.25×10^8	2.16×10^8	1.98×10^8	1.24×10^8
Cell growth rate (μ) – (0 – 40 h) (h^{-1})	0.034	0.037	0.033	0.022	0.036	0.038	0.036	0.020
Cell decay rate (K) – (40 – 80 h) (h^{-1})	0.008	0.008	0.005	0.011	0.006	0.006	0.005	0.006
Activity								
Initial specific activity (μmol/CFU)	1.16×10^{-9}	1.15×10^{-9}	1.16×10^{-9}	1.16×10^{-9}	1.12×10^{-9}	1.14×10^{-9}	1.10×10^{-9}	1.12×10^{-9}
Maximum specific activity (μmol/CFU)	3.03×10^{-9}	2.75×10^{-9}	2.56×10^{-9}	2.47×10^{-9}	2.64×10^{-9}	$2.52p \times 10^{-9}$	2.46×10^{-9}	2.19×10^{-9}
Specific TF increase rate (A) – (0 – 40 h)	0.028	0.047	0.028	0.016	0.031	0.029	0.024	0.021
Specific TF decrease rate (D) – (40 – 80 h)	0.010	0.009	0.012	0.011	0.001	0.002	0.003	0.016
Jadomycin								
Maximum jadomycin (AU 526nm)	0.495	0.558	0.485	0.248	0.332	0.174	0.435	0.412
Jadomycin Production rate (j) (h^{-1})	0.071	0.054	0.115	0.079	0.077	0.085	0.053	0.071

in a significant increase in jadomycin yield and was considered favourable for stressing *S.venezuelae.*

6.5. *Effect of Multiple Shock Treatment*

Ingram (1990) reported that addition of ethanol resulted in ethanol-induced leakage of the plasma membrane which affects the *S.venzuelae* growth, viability and metabolism. The author stated that the ethanol tolerance of growth in various bacteria tends to be a large part of the adaptive periods and is affected by the cell membrane composition. Growth of *S.venezuelae* sensitive cellular activities are subject to inhibition by ethanol stress, followed by cell survival, or loss of reproductive ability (Doull et al., 1994; Rix et al., 2004; Shapiro, 1958).

In this study, the effects of multiple ethanol shocks [once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h)] on the production of jadomycin in MSM production media were determined using ethanol concentration of 3 % v/v. The changes in the growth and activity of *S.venezuelae* under multiple shocks with ethanol are presented in Table 6.7.

6.5.1. *Streptomyces venezuelae Growth*

At the time of inoculations in the nutrient deprived amino acid rich MSM production media, the CFU/mL was 0.56×10^8, 0.58×10^8 and 0.55×10^8 CFU/mL and the corresponding triphenyl formazan yield was 0.063, 0.065 and 0.062 μmol for the cultures stressed with 3 % (v/v) ethanol once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively.

The cell number increased with time reaching a maximum of 1.54×10^8 CFU/mL at the 30 h, 1.16×10^8 CFU/mL at the 30 h, and 1.10×10^8 CFU/mL at the 20 h for the cultures shocked with 3 % (v/v) ethanol once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively. The corresponding TF yield also increased to a maximum of 0.463 μmol at the 20 h, 0.256 μmol at the 30 h, and 0.201 μmol at the 30 h for the cultures stressed with 3 % (v/v) ethanol once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively. The *S.venezuelae* grew exponentially post multiple ethanol shocks. The cell growth can be described by equation (6.2). The specific growth rate (μ) of the *S.venezuelae* population was

Table 6.7. Changes in growth, activity and jadomycin production of *S.venezuelae* as affected by 3 % v/v multiple ethanol shock.

Parameter	Multiple Ethanol Shock (3 % v/v)		
	(0 h)	(0, 3 h)	(0, 3 and 6 h)
Initial			
Cells (CFU/mL)	0.56×10^8	0.58×10^8	0.55×10^8
TF yield (μmol)	0.063	0.065	0.062
Specific TF (μmol/CFU)	1.12×10^{-9}	1.12×10^{-9}	1.12×10^{-9}
Maximum			
Cells(CFU/mL)	1.54×10^8	1.16×10^8	1.10×10^8
TF yield (μmol)	0.463	0.256	0.201
Specific TF (μmol/CFU)	3.04×10^{-9}	2.20×10^{-9}	1.92×10^{-9}
Final			
Cells (CFU/mL)	0.78×10^8	0.36×10^8	0.22×10^8
TF yield (μmol)	0.094	0.031	0.012
Specific TF (μmol/CFU)	1.23×10^{-9}	0.84×10^{-9}	0.52×10^{-9}
Jadomycin (OD_{526nm})	0.478	0.225	0.081

calculated from the slope of the linear portion of the growth curve after plotting ln (N_t/N_0) vs time. The specific growth rate (μ) measured in this study was 0.033, 0.029 and 0.023 h^{-1} for the cultures shocked with 3 % (v/v) ethanol once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively.

The cell number then declined after the first 20 - 30 h reaching 0.78×10^8, 0.36×10^8 and 0.22×10^8 CFU per mL at the end of the experiment (80 h) for the cultures shocked with 3 % (v/v) ethanol once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively. The corresponding final triphenyl formazan (TF) yield was 0.094 μmol, 0.031 μmol and 0.012 μmol for the cultures shocked with 3 % (v/v) ethanol once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively. The cell decay can be described by equation (6.3). The cell decay rate (k) of the S.venezuelae population was calculated from the slope of the linear portion of the population decay curve after plotting ln (N_t/N_m) vs time. The specific cell decay rate (k) measured in this study was 0.008, 0.014 and 0.020 h^{-1} for the cultures shocked with 3 % (v/v) ethanol at once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively.

The result showed that the addition of alcohol inhibited the growth and cell activity. This was a result of altering the transport system of amino acid permease, glucose uptake and biosynthesis mechanism within the cell (Bisson, 1999; Alexandre and Charpentier, 1998). The addition of ethanol at different times tends to affect the physiological state of bacteria and inhibit their cell growth. Addition of ethanol twice (0, 3 h) and three times (0, 3 and 6 h) reduced the cell number and TF yield compared with addition of ethanol once (0 h).

Sikkema et al. (1995) reported that the addition of organic solvents like ethanol leads to a specific permeabilization of the bacterial cell membranes. Several researchers found the addition of ethanol at different time periods to leads to different adverse effects such as release of potassium ions, interaction with ATP, denaturation of protein molecules and leakage of important macromolecules such as RNA and phospholipids (Heipieper et al., 1991; Isken and Bont, 1998).

Alexandre et al. (2001) reported that when the level of ethanol was increased, the proton inflow increased and resulted in the rapid disintegration of the electrochemical gradient

within the plasma membrane following rapid intracellular acidification. The authors found the addition of ethanol during different stages of growth caused adverse effects on the cells resulting in death and/or immediate spore transition.

Inoue and Horikoshi (1989) reported that the cell membrane is the main target for the toxic action caused by solvent like ethanol, therefore addition of solvents at different cell growth stages caused changes in membrane composition and played significant role in cell growth and activity.

The result also showed that shocking once (0 h) was favourable for the growth of *S.venezuelae*. In this study, multiple ethanol shocks, twice (0, 3 h) and three times (0, 3 and 6 h), were found to be causing extreme stress and inhibition of cell growth. Shocking the cells with ethanol once (0 h) resulted in more growth because the bacteria tend to develop a unique property of solvent tolerance as reported by Isken and Bont (1998). The results also indicated that the highest cell number and highest TF were not reached at the same time for all treatments.

Adaptation or tolerance mechanisms toward solvents are reported to take place in bacterial cell membrane by active excretion of solvents (Weber and de Bont, 1996; Vermue et al., 1993; George, 1996). When ethanol is added, the bacteria tend to repair membrane damage by enhanced phospholipid turnover or activating active efflux system to pump the solvent out of system (Ingram, 1990; Isken and Bont, 1998). Ethanol addition to the *S.venezuelae* cells is generally indicated by preferential distribution of ethanol to the hydrophobic environment of lipid bilayers, resulting in a disruption of membrane structure that adversely affects many membrane-associated processes (Rose, 1993; Lloyd et al., 1993; Jones, 1989).

Since the *S.venezuelae* cells are shocked in nutrient deprived production media, the addition of ethanol at multiple times affected their ability to uptake glucose, maltose, ammonium and amino acid and other vital nucleotides which caused reduced growth and activity (Serrano, 1991; Catwright et al., 1987 and Coote et al., 1991). Piper (1995) reported that ethanol addition leads to destabalization of the hydrophobic interactions within the native protein structures in *S.venezuelae* and exposes localized hydrophobic regions on the

proteins, leading to the association of chaperones with these destabilized proteins which may be the signal for activation of heat shock genes in bacteria.

Curran et al. (1994) and Gropper et al. (1993) reported that the more hydrophobic alkanols are better chemical/stress inducers of heat shock proteins in bacteria and result in the increased capacity to destabalize the hydrophobic interactions that maintain the protein conformations in cells.

6.5.2. *Streptomyces venezuelae Specific Activity*

At the time of inoculation of the nutrient deprived amino acid rich MSM production media, the specific activity was 1.12×10^{-9}, 1.12×10^{-9} and 1.12×10^{-9} μmol/CFU for the cultures to be shocked with 3 % v/v ethanol at once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively.

The specific activity of the *S.venezuelae* was noticed to increase greatly for the cultures that was ethanol shocked once (0 h) compared to those shocked twice (0, 3 h) and three times (0, 3 and 6 h), respectively. This shows that cell growth caused by multiple shocks resulted in decreases in cell growth and cell activity.

The specific activity increased with time reaches a maximum of 3.04×10^{-9}, 2.20×10^{-9} and 1.82×10^{-9} μmol/CFU at the 30 h for the cultures shocked with 3 % (v/v) ethanol once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively. The increase in specific activity followed a similar pattern to that of the cell growth. The increases in the specific activity indicated that dehydrogenase enzyme within the cells were active. The upward portion of the specific activity curve can be described equation (6.4). The rate of increase in the specific activity (A) was 0.034, 0.017 and 0.014 h^{-1} for the cultures shocked with 3 % (v/v) ethanol once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively.

After reaching the maximum growth, the specific activity declined reaching 1.23×10^{-9}, 0.84×10^{-9}, and 0.52×10^{-9} μmol/CFU at 80 for the cultures shocked with 3 % (v/v) ethanol once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively. The downward portion of the specific activity curve can be described by equation (6.5). The specific activity decay

rate (D) was 0.011, 0.012 and 0.016 h^{-1} for the cultures shocked with 3 % (v/v) ethanol once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively.

6.5.3. *Jadomycin Production*

The antibiotic production by *S.venezuelae* was promoted by exposure to ethanol. Although the process whereby alcohol shock treatment induces the production of jadomycin B has not been determined, Doull et al. (1994) reported on the ability of alcohol treatment to trigger a heat-shock response and effectively act as a source of polyketide precursors to promote the release of the secondary metabolites in *S.venezuelae*. There has been no report on the relation between the antibiotic production and multiple ethanol shocks

The result obtained from this study showed that addition of 3 % v/v ethanol once proved to be more effective in inducing the jadomycin production than the addition of ethanol twice (0, 3 h) and three times (0, 3 and 6 h). Shocking multiple times significantly affected the growth and secondary metabolite production than shocking with high temperature.

Exposure to ethanol once (0 h) causes the bacteria to produce heat shock proteins (HSP) which induced the production of jadomycin. Inducing the ethanol stress multiple times caused extreme protein denaturation and leakage of important macromolecules through the cell membrane making it not favoring for antibiotic production (Nikaido, 2003). A high concentration of alcohol denatures the cell protein by disrupting the side chain intra molecular hydrogen bonding (Piper, 1995). The most commonly seen effects are the ethanol inhibition on thick peptidoglycan cell wall (Piper, 1995). As a solvent, alcohol influences the cellular metabolic properties and functions by altering the environmental conditions in which the intercellular enzymes catalyze reactions.

The jadomycin B concentration increased with time reaching its maximum after 72 h post ethanol shock. Jadomycin production by *S.venezuelae* culture can be described by equation (6.6). The jadomycin production rate (P) was calculated from the slope of the linear portion of the jadomycin curve after plotting ln $(P_f - P_t)$ vs time. The jadomycin production rate (j) measured in this study was 0.116, 0.088 and 0.80 h^{-1} for the cultures shocked with 3 % (v/v) ethanol at once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h), respectively.

6.5.4. *Jadomycin Kinetics*

The jadomycin kinetic parameters are shown in Table 6.8. The culture shocked with 3 % v/v ethanol once (0 h) resulted in higher cell growth, TF yield and cell activity. The increase in number of shocks twice [(0, 3 h) and three times (0, 3 and 6 h)] caused inhibition in growth and activity of *S.venezuelae*. The 3 % v/v ethanol shock once (0 h) resulted in high jadomycin yield (similar to that of ethanol shock twice).

6.6. *Ethanol and Nutrient Shock*

Doull et al. (1994) reported that production media containing 75 mM produced maximum jadomycin (69 µg ml^{-1}), whereas the 45 and 60 mM concentration were slightly lesser in amount (63 and 66 µg ml^{-1}), respectively. They reported a presence of structurally related jadomycins metabolite based on differences in the colour of cultures grown in media with various amino acids. Rix et al. (2004) proposed the mechanism by which chemical insertion occur causing the polyketide-derived aldehyde intermediate to couple with amino acid (isoleucine) to give an aldimine, which after cyclization forms a unique oxazolone ring. Jakeman et al. (2005) reported that both natural and non-natural amino acids are incorporated into the jadomycin oxazolone ring. The aim of this work was to investigate the activity of *S.venezuelae* and production of secondary metabolites at varying isoleucine concentrations (45, 60 and 75 mM) with three ethanol concentration (3.0, 4.5 and 6.0 % v/v). Jakeman et al. (2006) reported that increasing the amino acid concentrations from 15 to 75 mM and shocking with 3 % v/v resulted in higher jadomycin B production although nitrogen concentrations above 45 mM titres did not change significantly. The change in the growth and activity of *S.venezuelae* at different nitrogen and ethanol concentrations are presented in Table 6.9.

6.6.1. *Streptomyces venezuelae Growth*

At the time of inoculations in the nutrient deprived amino acid rich MSM production media, the CFU/mL for the 3 % (v/v) ethanol shock was 0.38 x 10^8, 0.37 x 10^8 and 0.38 x 10^8 CFU/mL and the corresponding triphenyl formazan yield was 0.055, 0.056 and 0.057 µmol for the culture with 45, 60 and 75 mM nitrogen concentrations, respectively. For 4.5 % (v/v)

Table 6.8. Jadomycin kinetic parameters affected by multiple ethanol shock.

Parameters	Multiple ethanol (3 % v/v) shocking		
	(0 h)	(0 and 3 h)	(0, 3 and 6 h)
Growth			
Initial cell number (CFU/mL)	0.56×10^8	0.58×10^8	0.55×10^8
Maximum cell number (CFU/mL)	1.54×10^8	1.16×10^8	1.10×10^8
Cell growth rate (μ) – (0 – 30 h) (h^{-1})	0.033	0.029	0.023
Cell decay rate (k) – (30 – 80 h) (h^{-1})	0.008	0.014	0.020
Activity			
Initial specific activity (μmol/CFU)	1.12×10^{-9}	1.12×10^{-9}	1.12×10^{-9}
Maximum specific activity (μmol/CFU)	3.04×10^{-9}	2.20×10^{-9}	1.82×10^{-9}
Specific TF increase rate (A) – (0 – 30 h) (μmol/CFU)	0.034	0.017	0.014
Specific TF decrease rate (D) – (30 – 80 h) (μmol/CFU)	0.011	0.012	0.016
Jadomycin			
Maximum jadomycin (AU 526nm)	0.492	0.293	0.129
Jadomycin Production rate (j) (h^{-1})	0.116	0.088	0.080

Table 6.9. Changes in growth, activity and jadomycin production of *S.venezuelae* as affected by nitrogen variation and ethanol concentration.

Parameters	Nutrient Deprivation (mM)								
	3.0 % v/v			4.5 % v/v			6.0 % v/v		
	45 mM	60 mM	75 mM	45 mM	60 mM	75 mM	45 mM	60 mM	75 mM
Initial									
Cells (CFU/mL)	0.38×10^8	0.37×10^8	0.38×10^8	0.31×10^8	0.31×10^8	0.32×10^8	0.33×10^8	0.35×10^8	0.33×10^8
TF yield (μmol)	0.054	0.053	0.053	0.044	0.043	0.045	0.042	0.044	0.046
Specific TF (μmol/CFU)	1.42×10^{-9}	1.43×10^{-9}	1.39×10^{-9}	1.41×10^{-9}	1.38×10^{-9}	1.40×10^{-9}	1.35×10^{-9}	1.38×10^{-9}	1.39×10^{-9}
Maximum									
Cells(CFU/mL)	1.32×10^8	1.56×10^8	1.72×10^8	1.68×10^8	1.68×10^8	1.80×10^8	1.34×10^8	1.46×10^8	1.52×10^8
TF yield (μmol)	0.395	0.473	0.528	0.388	0.450	0.512	0.358	0.398	0.455
Specific TF (μmol/CFU)	2.99×10^{-9}	3.03×10^{-9}	3.06×10^{-9}	2.30×10^{-9}	2.67×10^{-9}	2.84×10^{-9}	2.67×10^{-9}	2.72×10^{-9}	2.99×10^{-9}
Final									
Cells (CFU/mL)	0.45×10^8	0.78×10^8	0.85×10^8	0.74×10^8	0.78×10^8	0.90×10^8	0.50×10^8	0.56×10^8	0.80×10^8
TF yield (μmol)	0.082	0.096	0.124	0.092	0.110	0.146	0.056	0.072	0.106
Specific TF (μmol/CFU)	1.23×10^{-9}	1.45×10^{-9}	1.82×10^{-9}	1.24×10^{-9}	1.41×10^{-9}	1.62×10^{-9}	1.12×10^{-9}	1.28×10^{-9}	1.32×10^{-9}
Jadomycin (OD_{526nm})	0.440	0.475	0.518	0.480	0.545	0.621	0.435	0.445	0.562

ethanol shocked culture, the CFU/mL was 0.31×10^8, 0.31×10^8 and 0.32×10^8 CFU/mL and the corresponding triphenyl formazan yield was 0.044, 0.043 and 0.045 μmol for the culture with 45, 60 and 75 mM nitrogen concentrations, respectively. For the culture shocked with 6 % (v/v) ethanol, the CFU/mL was 0.35×10^8, 0.35×10^8 and 0.33×10^8 CFU/mL and the corresponding triphenyl formazan yield was 0.042, 0.044 and 0.046 μmol for 45, 60 and 75 mM nitrogen concentrations, respectively.

Alcohol induction in *S.veneauelae* inhibits the growth and cell activity by altering general amino acid permease system, with in the cell (Bisson, 1999; Alexandre and Charpentier, 1998). However, presence of higher amino acid levels causes the bacteria to utilize the source and begin to grow. Jakeman et al. (2006) reported that an increase in amino acid concentration to 75 mM with 3 % v/v ethanol shock did not significantly improve the jadomycin B production but produced slightly higher levels than at 45 and 60 mM.

Untrau et al. (1994) reported that when isoleucine is utilized as a source of nitrogen, amino acids can generate ammonium or glutamate. A primary reaction of *Steptomyces* in the utilization of amino acids creates a partition of the molecule into the amino group on one side and the carbon backbone on the other side by means of deamination or transfer of amino acid to an acceptor. Voelker and Altaba (2001) reported that when the intracellular amino acid level is high, the nitrogen assimilation in *Streptomyces* precedes either by the GDH or ADH pathway. However, these exhibit high affinity constant values and results in poor assimilation of ammonium when the intracellular level of amino levels falls down to a threshold value. Bhatnagar et al. (1988) reported that growth restriction by limiting the nitrogen availability allows the *S.venezuelae* to permit the genetic information for secondary processes to be influenced by various supporting mechanisms.

In this study varying the nitrogen concentration from 45 mM to 75 mM plays a significant role in increasing the intensity of *S.venezuelae* growth. This occurs not only because limiting the supply of an essential nutrient is an effective method of restricting growth but also because they result in specific metabolic and regulatory effects. Vining and Doull (1988) reported that nutritional stress has not been linked to activation of heat shock proteins, except in molecular control mechanisms.

For the culture shocked with 3 % (v/v) ethanol concentration, the cell number increased with time reaching a maximum of 1.32×10^8 CFU/mL, 1.56×10^8 CFU/mL and 1.72×10^8 CFU/mL at 32 h for the culture containing 45, 60 and 75 mM nitrogen concentrations, respectively. The corresponding TF yield also increased to a maximum of 0.395 μmol, 0.473 μmol and 0.528 μmol for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively. For the culture shocked with 4.5 % (v/v) ethanol concentration, the cell number increased with time reaching a maximum of 1.68×10^8 CFU/mL at the 24 h, 1.68×10^8 CFU/mL at the 32 h and 1.80×10^8 CFU/mL at the 32 h for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively. The corresponding TF yield also increased to a maximum of 0.388 μmol, 0.450 μmol and 0.512 μmol for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively. For the culture shocked with 6 % (v/v) ethanol shock, the cell number increased reaching a maximum of 1.34×10^8 CFU/mL at the 32 h, 1.46×10^8 CFU/mL at the 32 h and 1.52×10^8 CFU/mL at the 32 h for the cultures containing 45, 60 and 75 mM nitrogen, respectively. The corresponding TF yield also increased to a maximum of 0.358, 0.398 and 0.455 μmol for the cultures containing 45, 60 and 75 mM nitrogen, respectively.

The growth of *S.venezuelae* post ethanol shock with varying nitrogen concentration appeared to be exponential. Thus, the cell growth can be described by equation (6.2). The specific growth rate (μ) of the *S.venezuelae* population was calculated from the slope of the linear portion of the growth curve after plotting ln (N_t/N_0) vs time. The specific growth rate (μ) measured in this study for 3 % (v/v) was 0.040, 0.047 and 0.055 h^{-1} for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively. The specific growth rate (μ) measured in this study for 4.5 % (v/v) was 0.051, 0.053 and 0.070 h^{-1} for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively. The specific growth rate (μ) measured in this study for 6 % (v/v) was 0.041, 0.044 and 0.050 h^{-1} for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively.

The uptake of the amino acids in the production media irrespective of concentrations is mainly dependent on the physiological state of the cells depending upon the concentration of ethanol added to shock *S.venezuelae* cells. A similar observation was also given by Langheinrich and Ring (1976) on *Streptomyces hydrogenans* cell growth. Alim and Ring

(1976) reported that the amino acid transport rates are less in the begining of lag and late stationary growth phases, and attain maximum values when the *S.venezuelae* cells are growing at maximum rate. Hence from this experiment, it was concluded that the amino acid uptake in the production medium is linked to the rate of macromolecular synthesis via endogenous mediators which control the activity and/ or the formation of transport limiting proteins. Ring et al. (1970) and Langheinrich and Ring (1976) reported that a pool of free intracellular amino acids are involved in regulation of active amino acid uptake providing negative feed- back signals. During the *S.venezuelae* cell growth in the production media the amino acid transport capacity increased and attained its highest level when the growth rate was maximum for all nitrogen and ethanol concentration.

The cell number then declined reaching 0.45×10^8, 0.78×10^8 and 0.85×10^8 CFU/mL at the end of the experiment (80 h) for the culture stressed with 3 % (v/v) ethanol concentration using 45, 60 and 75 mM nitrogen concentrations, respectively. The corresponding final triphenyl formazan (TF) yield was 0.082 μmol, 0.096 μmol and 0.124 μmol for the culture containing 45, 60 and 75 mM nitrogen concentrations, respectively. For the culture shocked with 4.5 % (v/v) ethanol, the cell number declined reaching 0.74×10^8, 0.78×10^8 and 0.90 $\times 10^8$ CFU per mL at the end of the experiment (80 h) for the cultures containing 45, 60 and 75 mM nitrogen concentration, respectively. The corresponding final triphenyl formazan (TF) yield was 0.092 μmol, 0.110 μmol, and 0.146 μmol for the culture containing 45, 60 and 75 mM nitrogen concentrations, respectively. For the culture shocked with 6 % (v/v) ethanol, the cell number then declined reaching 0.50×10^8, 0.56×10^8 and 0.80×10^8 CFU per mL at the end of the experiment (80 h) for the cultures containing 45, 60 and 75 mM nitrgen concentration, respectively. The corresponding final triphenyl formazan (TF) yield was 0.056 μmol, 0.072 μmol, and 0.106 μmol for the culture containing 45, 60 and 75 mM nitrogen concentration, respectively.

The cell decay can be described by equation (6.3). The cell decay rate (k) of the *S.venezuelae* population was calculated from the slope of the linear portion of the population decay curve after plotting ln (N_t / N_m) vs time. The specific growth rate (k) measured in this study for 3 % (v/v) was 0.013, 0.008 and 0.008 h^{-1} for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively. The specific growth rate (k) measured in this study for 4.5 %

(v/v) was 0.0012, 0.008 and 0.010 h^{-1} for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively. The specific growth rate (k) measured in this study for 6 % (v/v) was 0.015, 0.004 and 0.011 h^{-1} for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively.

During further growth in the production media the amino acid transport capacity decreased progressively resulting in minimal growth. According to Langheinrich and Ring (1976) the rate of growth depletion might be due to reduced transport activity and was observed when *S.venezuelae* cells shifted into the stationary growth phase after the absence of amino acids in production media. In the production phase, the levels of all amino acids decreased markedly and reached the lowest values at the end of the experiment. Jakeman et al. (2005) and Voelker and Altaba (2001) stated that size and the composition of the pool of free intracellular amino acids were undergoing substantial variations during the different growth phases of *S.venezuelae* cells. Langheinrich and Ring (1976) stated that active amino acid transport in *Streptomyces hydrogenans* growth is influenced by intracellular amino acids.

With an increase in the ethanol concentration to 6 % (v/v), the observed cell growth and activity is seen higher in 45 mM nitrogen concentration, because the cells are more stressed at this limiting situation. From various studies, it is evident that higher ethanol concentration and lower nutrient availability causes the inhibition of the cell growth by directly targeting the plasma membrane ATPase activity, the fluidity of which is modified during the alcohol stress. Alexandre et al. (2001) and Gasch et al. (2000) reported that during exposure to alcohol stress (ethanol) most of the down-regulated genes reflect cell growth arrest which occurs during different stress treatments causing the cell to save energy and adapt to new environmental conditions. These genes are involved in protein biosynthesis (34%), cell growth (4%), RNA metabolism (13%) and cellular biogenesis (3%).

Serrano (1991), Catwright et al. (1987) and Coote et al. (1991) reported that addition of the ethanol with increasing concentration will prevent the uptake of amino acid as well as cause leakage of these vital molecule from the cell wall causing reduced growth and activity when compared with less ethanol or more nitrogen concentrations. At the plasma membrane, the increased passive proton inflow results in dissipating the electrochemical potential

gradient maintained across the bacterial membrane and greatly affect the significant function of plasma membrane electrochemical gradient such as uptake of amino acids. Similarly, in the 4.5 % (v/v) ethanol shock, the cell growth and activity is seen higher at both 45 and 60 mM nitrogen concentrations. Whereas, in the shocked with ethanol concentration of 3 % (v/v), the cell growth and activity is maximum at higher nitrogen concentration.

6.6.2. *Streptomyces venezuelae Specific Activity*

At the time of inoculation in the nutrient deprived amino acid rich MSM production media stressed with 3 % (v/v) ethanol, the specific activity was 1.42×10^{-9}, 1.43×10^{-9}, and 1.39×10^{-9} μmol/CFU for the cultures containing 45, 60 and 75 mM nitrogen concentration, respectively. For culture shocked with 4.5 % (v/v) ethanol, the specific activity was 1.78×10^{-9}, 1.80×10^{-9}, and 1.77×10^{-9} μmol/CFU for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively. For the culture shocked with 6 % (v/v) ethanol, the specific TF was 1.35×10^{-9}, 1.38×10^{-9}, and 1.39×10^{-9} μmol/CFU μmol/CFU for the culture containing 45, 60 and 75 mM nitrogen concentrations, respectively.

The TF increased at a faster rate than that of the cell during the first 32 h, resulting in increased specific activity. For the culture shocked with 3 % (v/v) ethanol, the maximum specific TF was 2.99×10^{-9}, 3.03×10^{-9} and 3.06×10^{-9} μmol/CFU at the 32 h for the cultures containing 45, 60 and 75 mM nitrogen, respectively. For the cultures shocked with 4.5 % (v/v) ethanol, the maximum specific activity was 2.30×10^{-9}, 2.67×10^{-9} and 2.84×10^{-9} μmol/CFU at the 40 h for the cultures containing 45, 60 and 75 mM nitrogen, respectively. For the cultures shocked with 6 % (v/v) ethanol, the maximum specific activity was 2.67×10^{-9}, 2.72×10^{-9} and 2.99×10^{-9} μmol/CFU at the 32 h for the cultures containing 45, 60 and 75 mM nitrogen, respectively.

The highest specific activity of *S.venezuelae* was observed at 45 mM nitrogen concentration with 6 % v/v ethanol shock. The results indicated that the activity was affected by increases in nitrogen concentration of ethanol with optimal 60 mM nitrogen source. The increase in specific activity during the growth phase followed a similar pattern to that of the cell growth after alcohol shock. The upward portion of the specific activity curve can be described by equation (6.4). The specific activity (A) of the *S.venezuelae* population was

calculated from the slope of the linear portion of the growth curve after plotting ln (N_t/N_0) vs time. The specific activity (A) measured in this study for 3 % (v/v) was 0.018, 0.025 and 0.029 h^{-1} for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively. The specific activity (A) measured in this study for 4.5 % (v/v) was 0.009, 0.012, and 0.014 h^{-1} for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively. The specific growth rate (μ) measured in this study for 6 % (v/v) was 0.016, 0.019 and 0.022 h^{-1} for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively.

After 32 h, the TF yield declined at faster rate than that of the cells and as a result the specific TF declined. The final specific activity value for the cultures shocked with 3.0 % (v/v) ethanol was 1.23 x 10^{-9}, 1.45 x 10^{-9} and 1.82 x 10^{-9} µmol/CFU for the culture containing 45, 60 and 75 mM nitrogen, respectively. The final specific activity value for the cultures shocked with 4.5 % (v/v) ethanol was 1.24 x 10^{-9}, 1.41 x 10^{-9} and 1.62 x 10^{-9} µmol/CFU for the culture containing 45, 60 and 75 mM nitrogen, respectively. The final specific activity value for the cultures shocked with 6.0 % (v/v) ethanol was 1.12 x 10^{-9}, 1.28 x 10^{-9} and 1.32 x 10^{-9} µmol/CFU for the cultures containing 45, 60 and 75 mM nitrogen, respectively. The downward portion of the specific activity curve can be described by equation (6.5). The specific activity decay (D) of the *S.venezuelae* population was calculated from the slope of the linear portion of the growth curve after plotting ln (N_t/N_0) vs time. The specific TF decay (D) measured in this study for 3 % (v/v) was 0.0011, 0.006 and 0.009 h^{-1} for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively. The specific activity decay (D) measured in this study for 4.5 % (v/v) was 0.009, 0.009 and 0.001 h^{-1} for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively. The specific growth rate (μ) measured in this study for 6 % (v/v) was 0.011, 0.013 and 0.006 h^{-1} for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively.

6.6.3. *Jadomycin production*

Jadomycin is only produced when an additional stress is applied to nutritionally unbalanced cultures (Doull et al., 1994; Brooks et al., 2012 and Burdock et al., 2008). Brooks et al. (2012) reported that a gene within the cell is involved in jadomycin biosynthesis and has been found to affect the morphological differentiation in *S.venezuelae*. Jakeman et al. (2006) and Rix et al. (2004) reported those *Streptomyces* genuses are able to metabolize the

molecules (amino acid) in the production media extracellularly by hydrolytic enzymes. It is interesting to note that cultures maintained at varying concentration (45 and 75 mM) rather than the optimal concentration (60 mM) have effectively triggered the genes responsible for secondary metabolite production.

Shapiro (1989) studied that the antibiotic biosynthesis is correlated and controlled by activity that regulates the assimilation of nitrogen, glutamate dehydrogenase and glutamate synthase are the most favourable enzymes that prove the regulatory linkage. The reason for not choosing the concentration below 45 mM is because the production media is made of limited source of nutrients which make it difficult to cause transition and sufficient production of titers were not seen during shocking.

The jadomycin B concentration increased with time reaching a maximum after 72 h post heat shock for the cultures shocked with ethanol (3.0, 4.5 and 6 % v/v) containing 45, 60 and 75 mM nitrogen concentrations. The jadomycin B concentration increased with time reaching its maximum after 72 h post ethanol shock. Jadomycin production by *S.venezuelae* culture can be described by equation (6.6). The jadomycin production rate (P) was calculated from the slope of the linear portion of the jadomycin curve after plotting $\ln (P_f - P_t)$ vs time. The jadomycin production rate (j) measured in this study for 3 % (v/v) was 0.040, 0.067 and 0.069 h^{-1} for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively. The jadomycin production rate (j) measured in this study for 4.5 % (v/v) was 0.058, 0.071 and 0.102 h^{-1} for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively. The jadomycin production rate (j) measured in this study for 6 % (v/v) was 0.060, 0.084 and 0.097 h^{-1} for the cultures containing 45, 60 and 75 mM nitrogen concentrations, respectively.

Doull and Vining (1990) reported that the production of polyketide metabolites by nutrient limitation has been reported to influence antibiotic yields. Martin (1983) and Behal (1987) reported that phosphate depletion and nitrogen deletion resulted in the synthesis and production of polyene and tetracycline in *Streptomyces*. Dekleva et al. (1985) also reported that by limiting the presence of nutrient sources like phosphate, ammonia and readily assimilated carbon sources resulted in anthracyclines antibiotic production from *Streptomyces*.

Lubbe et al. (1985) reported that the process that limits the nitrogen source results in the suppression of secondary metabolic pathways by inhibiting vital key enzyme reactions in *Steptomyces*. Martin et al. (1988) reported that limiting the nitrogen source can also repress the synthesis of the secondary pathway enzymes in bacteria. The study indicated that this repression process occurred in *S.griseus* affecting the candicidin antibiotic production, from which a phosphate-regulated promoter sequence for the pab genes involved in candicidin synthesis has been cloned and prevented. Brana et al. (1986) reported that excess of nitrogen source (glutamate) repressed the antibiotic synthesis (cephalosphorin) in *S. clavuligerus* where inhibition by the nitrogen source was believed to be due to its effect on transport or metabolic activity within the cell.

Aharonowitz and Demain (1979) reported that increasing the level of nitrogen source favors the conditions for catabolism of certain nutrient molecules and enhance catabolic processes that increase the level of antibiotic precursors. This process influences the antibiotic biosynthesis mechanism and results in higher yield. However, limiting the level of nitrogen source could trigger the catabolism of protein molecules by protease enzymes and provides free amino acid precursors for the metabolic pool (Martin, 1977).

Aharonowitz (1980) reported that the presence of adequate concentrations of the amino donors, in this case isoleucine is crucial for the enzymes within the cell to operate and be synthesized. In *Streptomyces* species, the presence of a nitrogen source is assimilated by two metabolic pathways. So, when the concentration of nitrogen is low (45 mM), enzyme glutamate dehydrogenase (GDH) which has low affinity for nitrogen source catalyzes the ATP dependent procutin of glutamine from nitrogen source, whereas in the high nitrogen concentration (75 mM) they operate primarily targeting the mechanism to activate antibiotic biosynthesis. The enzyme glutamate dehydrogenase combined with enzyme glutamate synthase (GOGAT) favors the antibiotic synthetase for higher secondary metabolite production.

Aldehyde is believed to be the intermediate molecule that is incorporated with the amino acids. Initial amino acid condensation is caused by a series of sequences involving decarboxylation and ring-closing to produce the pentacyclic skeleton of jadomycin (Sahif et al., 2012). Yang et al. (1996) suggested that in the presence of excess amino acid nucleophile

(e.g., isoleucine) incorporation start with an intermolecular 1,4-addition across the quinone enone followed by a decarboxylation. Rohr et al. (1992) proposed that the amine of isoleucine condenses with the aldehyde to form an intermediate imine followed by subsequent carboxylate addition to the imine. Amine addition to the quinone and decarboxylation leads to the pentacyclic core of jadomycin. It is still an open question as to whether the amino acid incorporation proceeds through an enzymatic or a spontaneous process.

The production media with 75 mM isoleucine shocked with 4.5 % v/v concentration of ethanol tended to produce a significant amount of jadomycin. However, 45 mM isoleucine concentration with 4.5 % v/v ethanol proved to be effective in inducing jadomycin B production, thereby reducing the amount of isoleucine usage. As seen from the previous study, 4.5 % v/v ethanol in 60 mM isoleucine concentration was also effective in the alcohol experiment, however shocking with 45 mM isoleucine proved to be much more effective in inducing the jadomycin production.

The result suggested that media with 75mM produced considerably higher amount of titers and goes on increasing with smaller shock concentrations (3% and 4.5% v/v), but when the concentration is increased they had slight decline at the end. This show with ample supply of nitrogen sources, cells tend to utilize them to multiply, which cause more cell growth and more transition from vegetative cells to spore in order to facilitate the jadomycin production. This shows that even when the cells were exposed to higher alcohol concentration the transition from vegetative cells to spores was not completely 100% resulting in more jadomycin production and more growth patterns. In the 45 mm deprived condition, the cell growth was controlled due the limited availability of the nitrogen, which caused the *Streptomyces* to transition to spores more efficiently than at 75mM. A limiting nutrient supply of 45 mM along with shocking the cells with 4.5% v/v ethanol concentration resulted in more efficient *Streptomyces* transition from vegetative cells to spores than other shock condition.

6.6.4. *Jadomycin Kinetics*

The jadomycin kinetic parameters are shown in Table 6.10. The culture shocked with

Table 6.10. Jadomycin kinetic parameters affected by nitrogen variation and ethanol concentration.

Parameters	3.0 % v/v			4.5 % v/v			6.0 % v/v		
	45 mM	60 mM	75 mM	45 mM	60 mM	75 mM	45 mM	60 mM	75 mM
Growth									
Initial cell number (CFU/mL)	0.38×10^8	0.37×10^8	0.38×10^8	0.31×10^8	0.31×10^8	0.32×10^8	0.33×10^8	0.35×10^8	0.33×10^8
Maximum cell number (CFU/mL)	1.32×10^8	1.56×10^8	1.72×10^8	1.68×10^8	1.68×10^8	1.80×10^8	1.34×10^8	1.46×10^8	1.52×10^8
Cell growth rate (μ) – (0 – 56 h) (h^{-1})	0.040	0.047	0.055	0.050	0.053	0.070	0.041	0.044	0.050
Cell decay rate (K) – (56 – 80 h) (h^{-1})	0.013	0.008	0.008	0.012	0.008	0.010	0.015	0.004	0.011
Activity									
Initial specific activity (μmol/CFU)	1.42×10^{-9}	1.43×10^{-9}	1.39×10^{-9}	1.78×10^{-9}	1.80×10^{-9}	1.77×10^{-9}	1.35×10^{-9}	1.38×10^{-9}	1.39×10^{-9}
Maximum specific activity (μmol/CFU)	2.99×10^{-9}	3.03×10^{-9}	3.06×10^{-9}	2.30×10^{-9}	2.67×10^{-9}	2.84×10^{-9}	2.67×10^{-9}	2.72×10^{-9}	2.99×10^{-9}
Specific TF increase rate (A) – (0 – 56 h)	0.018	0.025	0.029	0.009	0.012	0.014	0.016	0.019	0.022
Specific TF decrease rate (D) – (56 – 80 h)	0.011	0.006	0.009	0.009	0.009	0.001	0.011	0.013	0.006
Jadomycin									
Initial jadomycin (AU 526nm)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Maximum jadomycin (AU 526nm)	0.440	0.495	0.498	0.485	0.558	0.621	0.460	0.485	0.562
Jadomycin Production rate (j) - (h^{-1})	0.040	0.067	0.069	0.058	0.071	0.102	0.060	0.084	0.097

3.0 % v/v ethanol resulted in higher cell growth, TF yield and cell activity with 75 mM nitrogen concentrations. In 4.5 % v/v ethanol shock, the more cell growth and TF yield was observed in 60 mM nitrogen concentration.When the shock concentration was increased to 6 % v/v, the cell growth and activity was observed higher at 60 mM. On the other hand, the culture with 75 mM nitrogen concentration produced more jadomycin irrespective of ethanol concentration, but 4.5 % v/v ethanol shock with 75 mM produced higher jadomycin yield. The results showed that the smaller nitrogen concentration and/or the higher ethanol concentration the longer the time needed for the cells to regrow and the lower the maximum cell number and cell activity.

6.7. *Comparative Analysis*

The kinetic parameters of various treatments (at their optimum conditions) are shown in Table 6.11. The results indicated that shocking the culture once with ethanol (3 % v/v) produced 81.5 % more jadomycin than shocking with heat at a temperature of 45°C for 1 h (0.492 AU$_{526nm}$ for ethanol compared to 0.271 AU$_{526nm}$ for heat shock. However, increasing the ethanol concentration to 4.5 % v/v produced 10.8 % more jadomycin than 3 % v/v ethanol (0.545 AU$_{526nm}$ for 4.5 % v/v ethanol compared to 0.492 AU$_{526nm}$ for 3 % v/v ethanol shock). Using nitrogen (75 mM) in the medium with the 4.5 % v/v ethanol further increased the jadomycin production by 13.9 % above that without nitrogen addition (0.621 AU$_{526nm}$ for nitrogen ethanol compared to 0.545 AU$_{526nm}$ without nitrogen). Therefore, the treatment with ethanol (4.5 % v/v) and nitrogen (75 mM) is recommended for jadomycin production.

Table 6.11. Kinetic parameters of various treatments at the optimum conditions.

Parameters	Optimum Treatment			
	Heat	Alcohol Type	No. of Shocks	Ethanol / Nutrient
	45ºC – 1 h	4.5 % v/v ethanol	One shock (0 h)	4.5 %v/v – 75mM N
Growth				
Maximum cell number (x 10^8 per mL)	1.82×10^8	1.80×10^8	1.54×10^8	1.80×10^8
Final cell number (x 10^8 per mL)	1.52×10^8	0.90×10^8	0.78×10^8	0.90×10^8
Cell growth rate (h^{-1})	0.023	0.037	0.033	0.053
Cell decay rate (h^{-1})	0.004	0.008	0.008	0.012
Activity				
Maximum specific activity (x 10^{-9} µmol/CFU)	2.26×10^{-9}	2.75×10^{-9}	3.04×10^{-9}	2.84×10^{-9}
Final specific activity (x 10^{-9} µmol/CFU)	1.68×10^{-9}	1.27×10^{-9}	1.23×10^{-9}	1.62×10^{-9}
Specific TF increase rate (h^{-1})	0.024	0.047	0.034	0.014
Specific TF decrease rate (h^{-1})	0.005	0.009	0.011	0.001
Jadomycin				
Maximum jadomycin (AU_{526nm})	0.271	0.545	0.492	0.621
Jadomycin Production rate (h^{-1})	0.118	0.054	0.116	0.102

Kinetic Parameters in Growth Medium

Specific growth rate = $0.19 \ h^{-1}$

Specific activity during exponential growth = 4.65×10^{-9} µmol/CFU

Specific activity during stationary phase = 4.34×10^{-9} µmol/CFU

Specific activity during lag phase = 3.85×10^{-9} μmol/CFU

CHAPTER 7. RECOMMENDATIONS

1. Optical density, cell number and dehydrogenase activity measurements were employed to measure the growth of *Streptomyces venezuelae* during growth in nutrient rich growth media and nutrient deprived amino rich MSM production media.The dehydrogenase activity correlated very well with the cell number and should be used to assess thecell growth and activity. Further work is needed to develop the assay for measuring spores.

2. The ability to successfully measure dehydrogenase activity of *Streptomyces venezuale* cells or spores in the MSM production media provided information about the state of the cells during the production of jadomycin under different environmental stress. Further work is needed to investigate the ability of spores to produce dehydrogenase enzymes, studying their morphology and activity using TEM and SEM microscope.

3. A maximum cell growth and faster cell transition from vegetative state to spores is a key to efficient jadomycin production by *S.venezuelae*. Efficient ethanol shock is important because it initiates the transition to spores. By achieving a higher and faster transition to spores through an optimized ethanol shock, higher yields of jadomycin B can be achieved more rapidly. Further work is needed to investigate the time required for cell to grow in the production media before external shock.

4. Further work is needed to develop and evaluate various techniques to measure and characterize jadomycin.

5. Further study is required to determine the pathway of jadomycin B production and whether it is produced intracellularly or extracellularly. By understanding these mechanisms a design may be developed to allow for the continuous production of jadomycin B, as opposed to batch methods.

CHAPTER 8. CONCLUSIONS

The application of environmental stress (heat and ethanol shock) is a vital step in stimulating the cells to produce jadomycin. Therefore, various shocking methods were performed effectively on *S.venezuelae* cell population to maximize jadomycin B production. The metabolic activity of the *S.venezuelae* is associated with their physiology and structure. The dehydrogenase activity (using tetrazolium salt) was employed for measuring *Streptomyces venezuelae* activity. The following conclusions can be made fron the study;

1. The result obtained from the growth experiments using nutrient rich MYM media showed that the temperature of 30 °C was favorable for the growth of *Streptomyces venezuelae.*

   (a) A lag period of 6 h was followed by an exponential growth period that lasted for 44 h (from the 6th h to 50th h). Following the exponential growth phase, a stationary growth period was observed from the 50th h till the end of the experiment (65 h).

   (b) The specific growth rate (μ) was 0.19 h^{-1}.

   (c) The activity of *S.venezuelae* during the lag phase (3.85 x 10^{-9} μmol/CFU) and during the stationary phase (4.34 x 10^{-9} μmol/CFU) was lower than that observed during exponential growth period (4.65 x 10^{-9} μmol/CFU).

2. The effect of the time of transferring the cells (18, 21 and 24 h) from the growth medium to the production medium on the growth of *S.venezuelae* and production of jadomycin was highly significant at the 0.001 level. Jadomycin production was initiated by shocking the healthy *S.venezuelae* cells after being transferred to the nutrient deprived amino acid rich MSM production media using 3 % v/v ethanol at a pH of 7 and temperature of 30 °C.

   (a) The culture that was transferred after 18 hour of growth in the growth medium resulted in higher cell number (1.42 x 10^{8} CFU/mL), TF yield (0.452 μmol) and cell activity (3.18 x 10^{-9} μmol/CFU) during the entire period of growth in the MSM production medium.

(b) The highest jadomycin (0.430 AU) was produced by the culture that was transferred after 18 h from the growth medium to the production medium.

3. The effects of heat shock treatment (35. 40, 45 and 50°C) of the *S.venezuelae* and the exposure time (0.5 and 1 h) on the growth of *S.venezuelae* and jadomycin production in the nutrient deprived amino acid rich MSM production media were significant at the 0.001 level. There was also a significant interaction between the temperature and time of exposure.

(a) The highest cell number and highest TF were not reached at the same time for all treatments.

(b) The culture that was heat shocked at 35°C for 0.5 h exposure time in the production medium resulted in higher cell number (2.04 x 10^8 CFU/mL), TF yield (0.558 µmol) and cell specific acitivity (2.86 x 10^{-9} µmol/CFU) during the entire period of growth in the MSM production medium.

(c) The highest jadomycin (0.271 AU) was produced by the culture that was heat shocked at a temperature of 40°C for 1 h.

(d) The cultures exposed to heat shock at the temperature 45°C started to produce jadomycin immediately after shocking.

4. The effects of alcohol type (ethanol and methanol) and alcohol concentration (3.0, 4.5, 6.0 and 7.5 % v/v) on *Streptomyces venezuelae* growth and jadomycin production in the nutrient deprived amino acid rich MSM production media were significant at the 0.001 level. There was also a significant interaction between the alcohol type and concentration at the 0.001 level.

(a) Ethanol shock produced more jadomycin than methanol.

(b) The highest cell number and highest TF were not reached at the same time for all treatments.

(c) The highest cell number of (2.25 x 10^8 and 1.77 x 10^8 CFU/mL) was produced with the alcohol concentrations of 3.0 and 4.5 % v/v for methanol and ethanol, respectively.

(d) The culture that was shocked with methanol at a concentration of 3 % v/v resulted in the highest cell number (2.25 x 10^8 CFU/mL), TF yield (0.595 µmol) and cell activity (2.64 x 10^{-9} µmol/CFU) during the entire period of growth in the MSM production medium.

5. The effects of multiple ethanol (3 % v/v) shocks [once (0 h), twice (0, 3 h) and three times (0, 3 and 6 h)] on the growth of *S.venezuelae* production of jadomycin in MSM production was highly significant at the 0.001 level.

(a) The highest cell number and highest TF were not reached at same time for all treatments.

(b) The culture shocked once (0 h) with 3 % (v/v) ethanol resulted in the highest cell number (1.54 x 10^8 CFU/mL), TF yield (0.463 µmol) and cell activity (3.04 x 10^{-9} µmol/CFU) during the entire period of growth in the MSM production medium.

(c) The increase in number of shocks [twice (0, 3 h) and three times (0, 3 and 6 h)] caused inhibition of growth and activity of *S.venezuelae*.

(d) The jadomycin B production was produced immediately after shocking the culture once (0 h) and after 10 h for cultures shocked twice (0, 3 h) and three times (0, 3 and 6 h).

(e) The highest jadomycin (0.492 AU) was produced by the culture that was shocked once (0 h).

6. The effects of ethanol concentration (3.0, 4.5 and 6.0 % v/v) and nitrogen (L-isoleucine) concentration (45, 60 and 75 mM) on the growth of *S.venezuelae* and production of jadomycin in the nutrient deprived amino acid rich MSM production media were highly significant at the 0.001 level. There was also a significant interaction between the parameters at the 0.001 level.

(a) The culture that was shocked with 4.5 % (v/v) ethanol and contained 75 mM nitrogen produced the highest cell number (1.80×10^8 CFU/mL), TF yield (0.512 μmol) and cell activity (2.84×10^{-9} μmol/CFU) during the entire period of growth in the MSM production medium.

(b) The jadomycin B was noted to increase slowly for the cultures shocked with 3.0 % (v/v) ethanol compared to the cultures shocked with 4.5 and 6.0 % (v/v) ethanol.

(c) The highest jadomycin (0.621 AU) was achieved by the culture that was shocked with 4.5 % (v/v) ethanol and contained 75 mM nitrogen concentration.

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Appendix A. S.venezuelae Growth

Table A.1.   *S.venezuelae* growth and dehydrogenase activity Trials measured through triphenyl formazan (TF) yield in MYM broth.

Time (h)	TF Yield (µmol)					CFU/ mL				
	Trial 1	Trail 2	Trial 3	Avg	SD	Trial 1	Trail 2	Trial 3	Avg	SD
0	0.033	0.030	0.031	0.031	±0.00	0.12×10^{10}	0.06×10^{10}	0.06×10^{10}	0.08×10^{10}	±3.46
4	0.033	0.030	0.031	0.031	±0.00	0.12×10^{10}	0.07×10^{10}	0.06×10^{10}	0.08×10^{10}	±3.21
6	0.036	0.038	0.039	0.038	±0.00	0.15×10^{10}	0.08×10^{10}	0.08×10^{10}	0.10×10^{10}	±4.04
8	0.063	0.065	0.074	0.067	±0.01	0.18×10^{10}	0.15×10^{10}	0.12×10^{10}	0.15×10^{10}	±3.00
10	0.090	0.078	0.085	0.084	±0.01	0.21×10^{10}	0.19×10^{10}	0.15×10^{10}	0.18×10^{10}	±3.06
12	0.113	0.105	0.109	0.109	±0.00	0.27×10^{10}	0.21×10^{10}	0.24×10^{10}	0.24×10^{10}	±3.00
14	0.186	0.178	0.190	0.184	±0.01	0.51×10^{10}	0.31×10^{10}	0.38×10^{10}	0.40×10^{10}	±10.1
16	0.325	0.302	0.318	0.315	±0.01	0.74×10^{10}	0.65×10^{10}	0.66×10^{10}	0.68×10^{10}	±4.93
18	0.405	0.425	0.431	0.420	±0.01	0.94×10^{10}	0.89×10^{10}	0.92×10^{10}	0.92×10^{10}	±2.52
20	0.504	0.490	0.487	0.494	±0.01	1.28×10^{10}	0.95×10^{10}	0.94×10^{10}	1.06×10^{10}	±19.3
24	0.582	0.608	0.623	0.605	±0.02	1.46×10^{10}	1.22×10^{10}	1.21×10^{10}	1.30×10^{10}	±14.1
30	0.705	0.693	0.726	0.708	±0.02	1.69×10^{10}	1.42×10^{10}	1.47×10^{10}	1.52×10^{10}	±14.3
35	0.743	0.738	0.816	0.765	±0.04	1.85×10^{10}	1.53×10^{10}	1.58×10^{10}	1.65×10^{10}	±17.2
40	0.778	0.768	0.842	0.796	±0.04	1.90×10^{10}	1.65×10^{10}	1.66×10^{10}	1.74×10^{10}	±14.1
45	0.804	0.815	0.871	0.830	±0.04	1.98×10^{10}	1.82×10^{10}	1.78×10^{10}	1.86×10^{10}	±10.5
50	0.830	0.822	0.889	0.847	±0.04	2.08×10^{10}	1.89×10^{10}	1.80×10^{10}	1.92×10^{10}	±14.2
55	0.864	0.853	0.900	0.872	±0.02	2.15×10^{10}	1.93×10^{10}	1.86×10^{10}	1.98×10^{10}	±15.1
60	0.837	0.831	0.838	0.835	±0.00	2.16×10^{10}	1.85×10^{10}	1.81×10^{10}	1.94×10^{10}	±19.1
65	0.821	0.830	0.833	0.828	±0.01	2.12×10^{10}	1.83×10^{10}	1.80×10^{10}	1.92×10^{10}	±17.6

Avg – Average for all trials
SD- Standard deviation for all trials

Appendix B Time of Culture Transfer into Production Medium

Table B 1. Trials for 18 h culture transfer into production medium.

Time (h)	TF Yield (μmol)					CFU/mL x 10^6					OD 526 nm (AU)				
	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.030	0.034	0.031	0.026	±0.002	024	026	025	025	±1.001	0.000	0.000	0.000	0.000	±0.000
8	0.152	0.162	0.161	0.108	±0.005	082	076	090	082	±7.021	0.022	0.036	0.033	0.030	±0.007
16	0.318	0.316	0.305	0.256	±0.007	114	114	120	116	±3.465	0.111	0.141	0.135	0.128	±0.016
24	0.425	0.419	0.410	0.375	±0.007	128	135	142	135	±7.006	0.195	0.250	0.214	0.219	±0.027
32	0.453	0.454	0.449	0.412	±0.002	136	146	144	142	±5.298	0.310	0.322	0.302	0.312	±0.010
48	0.319	0.305	0.323	0.278	±0.009	133	133	125	130	±4.614	0.373	0.408	0.41	0.398	±0.020
72	0.161	0.150	0.155	0.125	±0.005	096	128	120	114	±6.612	0.398	0.433	0.459	0.430	±0.030

Avg – Average for all trials
SD- Standard deviation for all trials

Table B 2. Trials for 21 h culture transfer into production medium.

Time	TF Yield (µmol)					CFU/mL x 10^6					OD 526 nm (AU)				
(h)	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.029	0.029	0.026	0.028	±0.001	019	025	022	022	±3.000	0.000	0.000	0.000	0.000	±0.000
8	0.152	0.128	0.117	0.116	±0.017	068	075	070	071	±3.605	0.044	0.052	0.048	0.048	±0.004
16	0.297	0.300	0.295	0.297	±0.002	103	115	112	110	±6.246	0.110	0.114	0.121	0.115	±0.005
24	0.394	0.415	0.403	0.404	±0.010	128	131	146	135	±9.643	0.192	0.192	0.196	0.192	±0.003
32	0.420	0.427	0.423	0.433	±0.003	130	134	150	138	±10.58	0.276	0.28	0.274	0.276	±0.003
48	0.285	0.296	0.305	0.295	±0.010	120	120	135	125	±8.660	0.414	0.339	0.395	0.382	±0.039
72	0.139	0.144	0.155	0.146	±0.007	099	113	124	112	±12.53	0.442	0.367	0.461	0.423	±0.049

Avg – Average for all trials
SD- Standard deviation for all trials

Table B 3. Trials for 24 h culture transfer into production medium.

Time	TF Yield (μmol)					CFU/mL x 10^6					OD 526 nm (AU)				
(h)	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.025	0.026	0.028	0.032	±0.001	015	022	023	020	±4.350	0.000	0.000	0.000	0.000	±0.000
8	0.118	0.121	0.116	0.158	±0.026	056	064	066	062	±5.291	0.021	0.017	0.022	0.020	±0.002
16	0.276	0.250	0.246	0.312	±0.001	096	106	110	104	±7.211	0.078	0.062	0.056	0.065	±0.011
24	0.373	0.376	0.377	0.418	±0.001	124	128	132	128	±4.000	0.166	0.151	0.158	0.158	±0.007
32	0.410	0.414	0.416	0.452	±0.002	132	126	150	136	±12.49	0.226	0.248	0.264	0.246	±0.019
48	0.281	0.282	0.273	0.315	±0.005	118	124	124	122	±3.464	0.356	0.342	0.338	0.345	±0.009
72	0.124	0.129	0.128	0.155	±0.002	096	102	114	104	±9.165	0.404	0.387	0.396	0.394	±0.008

Avg – Average for all trials
SD- Standard deviation for all trials

Appendix C. Heat Shock treatment (35, 40, 45 and 50 ºC) on Streptomyces venezeuale to initiate jadomycin B production in MSM production media.

Table C 1. Trials for 35 ºC heat shock treatment for 0.5 h exposure in MSM production medium.

Time	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
(h)	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.074	0.071	0.074	0.073	±0.002	075	071	070	072	±0.707	0.000	0.000	0.000	0.000	±0.000
8	0.249	0.259	0.260	0.256	±0.006	154	154	160	156	±4.243	0.010	0.000	0.001	0.000	±0.006
16	0.367	0.374	0.375	0.372	±0.005	172	176	168	172	±5.657	0.000	0.000	0.001	0.000	±0.001
24	0.444	0.455	0.467	0.455	±0.012	178	179	186	181	±4.950	0.007	0.001	0.010	0.006	±0.005
32	0.517	0.512	0.508	0.512	±0.005	190	190	196	192	±4.243	0.017	0.012	0.014	0.013	±0.003
40	0.537	0.556	0.517	0.536	±0.019	191	195	199	195	±2.828	0.016	0.021	0.017	0.018	±0.003
48	0.559	0.571	0.547	0.558	±0.012	195	204	198	199	±4.243	0.021	0.027	0.029	0.026	±0.004
56	0.540	0.549	0.537	0.542	±0.006	203	201	208	204	±4.950	0.035	0.038	0.042	0.037	±0.004
64	0.519	0.507	0.517	0.514	±0.007	204	198	198	200	±0.000	0.045	0.035	0.044	0.041	±0.006
72	0.440	0.446	0.441	0.442	±0.003	185	189	190	188	±0.707	0.045	0.049	0.040	0.044	±0.005
80	0.391	0.385	0.381	0.385	±0.005	172	177	179	176	±1.414	0.039	0.043	0.048	0.042	±0.005

Avg – Average for all trials
SD- Standard deviation for all trials

Table C 2. Trials for 40 ºC heat shock treatment for 0.5 h exposure in MSM production medium.

Time	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
(h)	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.070	0.071	0.074	0.071	±0.002	065	069	073	065	±2.828	0.000	0.000	0.000	0.00	±0.000
8	0.241	0.233	0.232	0.235	±0.005	127	138	140	127	±1.414	0.012	0.019	0.01	0.01	±0.005
16	0.313	0.308	0.317	0.312	±0.005	152	155	158	152	±2.121	0.056	0.074	0.062	0.064	±0.009
24	0.359	0.359	0.363	0.362	±0.002	166	162	176	166	±9.899	0.129	0.137	0.142	0.136	±0.007
32	0.410	0.420	0.416	0.415	±0.005	166	175	175	166	±0.000	0.184	0.176	0.173	0.178	±0.006
40	0.461	0.466	0.461	0.462	±0.003	182	186	196	182	±7.071	0.204	0.214	0.195	0.204	±0.010
48	0.458	0.452	0.452	0.453	±0.003	185	194	185	185	±6.364	0.217	0.235	0.211	0.221	±0.012
56	0.449	0.438	0.444	0.441	±0.006	193	200	195	193	±3.536	0.236	0.256	0.224	0.238	±0.016
64	0.433	0.426	0.416	0.424	±0.009	194	188	188	194	±0.000	0.233	0.256	0.237	0.242	±0.012
72	0.398	0.401	0.397	0.398	±0.002	182	179	194	182	±10.60	0.245	0.259	0.235	0.246	±0.012
80	0.357	0.351	0.349	0.352	±0.004	160	171	173	160	±1.414	0.238	0.254	0.215	0.235	±0.020

Avg – Average for all trials
SD- Standard deviation for all trials

Table C 3. Trials for 45 ºC heat shock treatment for 0.5 h exposure in MSM production medium.

Time	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
(h)	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.072	0.073	0.076	0.073	±0.002	075	068	073	072	±3.606	0.000	0.000	0.000	0.000	±0.000
8	0.155	0.155	0.158	0.155	±0.002	127	118	121	122	±4.583	0.01	0.011	0.015	0.012	±0.003
16	0.236	0.228	0.234	0.232	±0.004	132	135	138	134	±3.000	0.019	0.024	0.023	0.022	±0.003
24	0.283	0.286	0.288	0.285	±0.002	146	141	139	142	±3.606	0.086	0.092	0.097	0.091	±0.006
32	0.333	0.340	0.329	0.332	±0.006	156	155	157	156	±1.000	0.117	0.123	0.124	0.121	±0.004
40	0.358	0.351	0.359	0.356	±0.004	165	159	168	164	±4.583	0.203	0.212	0.197	0.204	±0.008
48	0.333	0.334	0.335	0.334	±0.001	155	162	169	162	±7.000	0.297	0.265	0.272	0.278	±0.017
56	0.280	0.282	0.284	0.282	±0.002	193	200	195	156	3.6060	0.315	0.306	0.315	0.312	±0.005
64	0.212	0.211	0.215	0.212	±0.002	144	157	149	150	±6.557	0.317	0.328	0.304	0.325	±0.012
72	0.180	0.190	0.162	0.177	±0.014	142	149	144	145	±3.606	0.339	0.335	0.321	0.331	±0.009
80	0.133	0.150	0.121	0.134	±0.014	127	150	143	140	±11.79	0.328	0.331	0.326	0.328	±0.003

Avg – Average for all trials
SD- Standard deviation for all trials

Table C 4. Trials for 50 ºC heat shock treatment for 0.5 h exposure in MSM production medium.

Time	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
(h)	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.074	0.074	0.074	0.073	±0.000	073	069	071	071	±2.000	0.000	0.000	0.000	0.000	±0.000
8	0.085	0.086	0.084	0.085	±0.001	091	085	082	086	±4.583	0.011	0.006	0.007	0.004	±0.004
16	0.108	0.142	0.134	0.127	±0.018	110	91	105	102	±9.849	0.037	0.021	0.022	0.018	±0.006
24	0.140	0.173	0.176	0.162	±0.020	116	112	117	115	±2.646	0.053	0.036	0.035	0.035	±0.001
32	0.161	0.206	0.201	0.189	±0.025	124	115	115	118	±5.196	0.085	0.052	0.064	0.056	±0.007
40	0.155	0.204	0.198	0.185	±0.026	120	124	119	121	±2.646	0.121	0.082	0.085	0.085	±0.002
48	0.127	0.131	0.134	0.130	±0.004	133	132	119	128	±7.810	0.14	0.132	0.128	0.127	±0.006
56	0.114	0.117	0.118	0.116	±0.002	142	136	121	133	±10.81	0.154	0.146	0.151	0.145	±0.006
64	0.093	0.095	0.098	0.095	±0.003	116	110	110	112	±3.464	0.156	0.166	0.149	0.156	±0.009
72	0.082	0.085	0.088	0.083	±0.003	104	099	091	098	±6.557	0.152	0.169	0.151	0.158	±0.009
80	0.063	0.069	0.068	0.066	±0.003	087	085	080	084	±3.606	0.156	0.161	0.152	0.155	±0.005

Avg – Average for all trials
SD- Standard deviation for all trials

Table C 5. Trials for 35 ºC heat shock treatment for 1.0 h exposure in MSM production medium.

Time (h)	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.072	0.071	0.073	0.071	±0.001	65	74	71	70	±2.121	0.000	0.000	0.000	0.000	±0.000
8	0.251	0.240	0.238	0.242	±0.007	146	155	143	148	±8.485	0.000	0.008	0.002	0.004	±0.004
16	0.313	0.314	0.348	0.324	±0.020	162	175	158	165	±12.02	0.000	0.018	0.015	0.011	±0.010
24	0.397	0.394	0.396	0.395	±0.001	178	175	178	177	±2.121	0.007	0.016	0.019	0.014	±0.006
32	0.442	0.448	0.447	0.445	±0.003	179	188	185	184	±2.121	0.026	0.021	0.025	0.024	±0.003
40	0.476	0.472	0.478	0.475	±0.003	188	181	201	190	±14.14	0.037	0.043	0.047	0.042	±0.005
48	0.534	0.513	0.525	0.524	±0.010	190	200	195	195	±3.536	0.043	0.048	0.052	0.048	±0.005
56	0.508	0.516	0.514	0.512	±0.004	197	197	197	197	±0.000	0.052	0.065	0.049	0.055	±0.009
64	0.453	0.457	0.466	0.458	±0.007	184	188	192	188	±2.828	0.065	0.052	0.058	0.058	±0.007
72	0.407	0.430	0.439	0.425	±0.017	181	176	180	179	±2.828	0.067	0.058	0.058	0.061	±0.005
80	0.370	0.380	0.373	0.374	±0.005	169	182	159	170	±16.26	0.063	0.058	0.071	0.060	±0.007

Avg – Average for all trials
SD- Standard deviation for all trials

Table C 6. Trials for 40 ºC heat shock treatment for 1.0 h exposure in MSM production medium.

Time	TF yield (μmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
(h)	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.074	0.071	0.073	0.072	±0.002	064	079	070	071	±6.364	0.000	0.000	0.000	0.00	±0.000
8	0.175	0.175	0.165	0.171	±0.006	119	128	128	125	±0.000	0.066	0.069	0.042	0.031	±0.015
16	0.283	0.277	0.284	0.281	±0.004	143	140	150	144	±7.071	0.094	0.097	0.085	0.092	±0.006
24	0.348	0.349	0.354	0.35	±0.003	156	152	166	158	±9.899	0.179	0.136	0.176	0.164	±0.024
32	0.404	0.392	0.394	0.396	±0.007	159	170	169	166	±0.707	0.289	0.245	0.255	0.221	±0.023
40	0.408	0.415	0.417	0.412	±0.002	180	180	186	182	±4.243	0.263	0.263	0.244	0.257	±0.011
48	0.397	0.401	0.400	0.398	±0.009	175	184	175	178	±6.364	0.252	0.244	0.247	0.268	±0.005
56	0.374	0.381	0.362	0.372	±0.005	168	191	166	175	±17.67	0.248	0.238	0.251	0.271	±0.007
64	0.348	0.338	0.342	0.342	±0.005	166	185	159	170	±18.38	0.264	0.276	0.266	0.268	±0.006
72	0.323	0.322	0.311	0.318	±0.007	170	162	169	167	±4.950	0.239	0.275	0.282	0.262	±0.023
80	0.258	0.265	0.248	0.256	±0.009	148	150	158	152	±5.657	0.262	0.254	0.26	0.258	±0.006

Avg – Average for all trials
SD- Standard deviation for all trials

Table C 7. Trials for 45 ºC heat shock treatment for 1.0 h exposure in MSM production medium.

Time (h)	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.075	0.071	0.073	0.073	±0.002	077	070	063	070	±7.000	0.000	0.000	0.000	0.000	±0.000
8	0.114	0.119	0.120	0.117	±0.003	116	110	119	115	±4.583	0.024	0.037	0.034	0.032	±0.007
16	0.180	0.186	0.181	0.182	±0.003	130	121	115	122	±7.550	0.031	0.054	0.059	0.048	±0.015
24	0.250	0.244	0.246	0.246	±0.005	136	134	135	135	±1.000	0.076	0.089	0.092	0.086	±0.009
32	0.288	0.279	0.289	0.285	±0.004	147	138	135	140	±6.245	0.112	0.115	0.125	0.117	±0.007
40	0.283	0.279	0.274	0.278	±0.003	130	130	154	138	±13.85	0.158	0.166	0.162	0.162	±0.004
48	0.266	0.269	0.264	0.266	±0.013	125	125	137	129	±6.928	0.171	0.211	0.226	0.203	±0.028
56	0.239	0.241	0.217	0.232	±0.002	120	123	117	120	±3.000	0.232	0.235	0.237	0.235	±0.003
64	0.180	0.177	0.180	0.178	±0.002	097	120	119	112	±13.00	0.274	0.241	0.249	0.255	±0.017
72	0.155	0.151	0.152	0.152	±0.015	111	108	096	105	±7.937	0.286	0.268	0.286	0.276	±0.010
80	0.108	0.111	0.106	0.108	±0.003	097	104	093	098	±5.568	0.294	0.28	0.274	0.282	±0.010

Avg – Average for all trials
SD- Standard deviation for all trials

Table C 8. Trials for 50 ºC heat shock treatment for 1.0 h exposure in MSM production medium.

Time (h)	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.075	0.071	0.073	0.073	±0.002	073	069	071	072	±2.000	0.000	0.000	0.000	0.000	±0.000
8	0.081	0.077	0.074	0.077	±0.003	061	068	067	065	±3.786	0.006	0.004	0.001	0.003	±0.003
16	0.101	0.121	0.127	0.116	±0.014	090	091	085	088	±3.215	0.011	0.009	0.009	0.009	±0.001
24	0.124	0.128	0.143	0.131	±0.010	116	102	098	105	±9.452	0.086	0.095	0.120	0.058	±0.018
32	0.128	0.122	0.141	0.130	±0.009	124	110	104	112	±10.26	0.122	0.116	0.116	0.098	±0.003
40	0.106	0.124	0.122	0.117	±0.010	115	106	093	104	±11.06	0.113	0.108	0.115	0.112	±0.004
48	0.104	0.103	0.107	0.104	±0.002	105	092	096	097	±6.658	0.12	0.108	0.117	0.115	±0.006
56	0.077	0.091	0.093	0.085	±0.009	092	086	079	085	±6.506	0.125	0.116	0.122	0.121	±0.005
64	0.062	0.083	0.079	0.074	±0.011	088	074	069	077	±9.849	0.128	0.136	0.138	0.125	±0.005
72	0.058	0.082	0.078	0.072	±0.013	078	076	071	075	±3.606	0.147	0.144	0.146	0.133	±0.002
80	0.041	0.065	0.062	0.055	±0.013	067	069	074	070	±3.606	0.166	0.152	0.151	0.135	±0.008

Avg – Average for all trials
SD- Standard deviation for all trials

Table C.9. The activity of *S.venezuelae* as measured by specific TF yield for all heat shock temperatures.

Time (h)	35ºC Shock		40ºC Shock		45ºC Shock		50ºC Shock	
	0.5 hour	1 hour	0.5 hour	1 hour	0.5 hour	1 hour	0.5 hour	1 hour
0	1.01×10^{-9}	1.01×10^{-9}	1.02×10^{-9}	1.01×10^{-9}	1.01×10^{-9}	1.04×10^{-9}	1.02×10^{-9}	1.01×10^{-9}
8	1.64×10^{-9}	1.63×10^{-9}	1.74×10^{-9}	1.36×10^{-9}	1.27×10^{-9}	1.01×10^{-9}	0.98×10^{-9}	1.18×10^{-9}
16	2.16×10^{-9}	1.96×10^{-9}	2.01×10^{-9}	1.95×10^{-9}	1.73×10^{-9}	1.49×10^{-9}	1.24×10^{-9}	2.00×10^{-9}
24	2.51×10^{-9}	2.23×10^{-9}	2.15×10^{-9}	2.22×10^{-9}	2.00×10^{-9}	1.82×10^{-9}	1.40×10^{-9}	1.24×10^{-9}
32	2.66×10^{-9}	2.41×10^{-9}	2.41×10^{-9}	2.38×10^{-9}	2.12×10^{-9}	1.96×10^{-9}	1.56×10^{-9}	1.16×10^{-9}
40	2.74×10^{-9}	2.50×10^{-9}	2.45×10^{-9}	2.71×10^{-9}	2.17×10^{-9}	2.14×10^{-9}	1.56×10^{-9}	1.12×10^{-9}
48	2.86×10^{-9}	2.68×10^{-9}	2.40×10^{-9}	2.23×10^{-9}	2.06×10^{-9}	2.26×10^{-9}	1.01×10^{-9}	1.07×10^{-9}
56	2.65×10^{-9}	2.59×10^{-9}	2.25×10^{-9}	2.12×10^{-9}	1.80×10^{-9}	2.12×10^{-9}	0.87×10^{-9}	1.00×10^{-9}
64	2.57×10^{-9}	2.43×10^{-9}	2.23×10^{-9}	2.01×10^{-9}	1.41×10^{-9}	1.58×10^{-9}	0.86×10^{-9}	0.96×10^{-9}
72	2.35×10^{-9}	2.37×10^{-9}	2.15×10^{-9}	1.90×10^{-9}	1.22×10^{-9}	1.44×10^{-9}	0.84×10^{-9}	0.96×10^{-9}
80	2.18×10^{-9}	2.20×10^{-9}	2.09×10^{-9}	1.68×10^{-9}	0.95×10^{-9}	1.26×10^{-9}	0.78×10^{-9}	0.78×10^{-9}

Appendix D. Alcohol (ethanol and methanol) Shock treatment using various concentrations (3, 4.5, 6 and 7.5 % v/v) on Streptomyces venezeuale to initiate jadomycin B production in MSM production media.

Table D 1. Trials for 3.0 % v/v ethanol shock treatment in MSM production medium.

Time (h)	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.072	0.070	0.074	0.072	±0.002	061	059	068	062	±4.726	0.000	0.000	0.000	0.000	±0.000
8	0.158	0.170	0.179	0.169	±0.010	075	069	074	072	±3.215	0.086	0.084	0.092	0.087	±0.004
16	0.333	0.325	0.331	0.330	±0.004	130	115	121	122	±7.550	0.235	0.228	0.245	0.236	±0.009
24	0.413	0.437	0.445	0.432	±0.017	147	149	136	144	±7.000	0.345	0.316	0.346	0.335	±0.017
32	0.450	0.464	0.462	0.458	±0.008	156	151	146	151	±5.000	0.378	0.396	0.382	0.385	±0.009
40	0.407	0.432	0.407	0.415	±0.014	151	142	139	144	±6.245	0.416	0.435	0.442	0.425	±0.013
48	0.348	0.299	0.361	0.336	±0.032	136	114	125	125	±11.00	0.466	0.462	0.451	0.46	±0.008
56	0.264	0.253	0.257	0.258	±0.005	118	098	114	110	±10.58	0.476	0.474	0.477	0.475	±0.002
64	0.193	0.180	0.192	0.188	±0.007	110	091	105	102	±9.849	0.498	0.496	0.492	0.495	±0.003
72	0.137	0.120	0.142	0.133	±0.011	097	089	090	092	±4.359	0.487	0.483	0.498	0.488	±0.008
80	0.103	0.104	0.084	0.096	±0.011	084	074	076	078	±5.292	0.482	0.479	0.466	0.475	±0.009

Avg – Average for all trials
SD- Standard deviation for all trials

Table D 2. Trials for 4.5 % v/v ethanol shock treatment in MSM production medium.

Time (h)	TF yield (μmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.076	0.078	0.072	0.072	±0.003	066	059	070	065	±5.568	0.000	0.000	0.000	0.000	±0.000
8	0.161	0.174	0.176	0.17	±0.008	070	080	090	080	±10.00	0.158	0.143	0.169	0.156	±0.013
16	0.343	0.329	0.324	0.33	±0.010	125	135	142	134	±8.544	0.327	0.308	0.288	0.316	±0.020
24	0.464	0.456	0.468	0.462	±0.006	166	162	176	168	±7.211	0.412	0.395	0.366	0.388	±0.023
32	0.486	0.471	0.506	0.487	±0.018	176	180	175	177	±2.646	0.442	0.478	0.448	0.456	±0.019
40	0.457	0.472	0.474	0.47	±0.009	172	176	177	175	±2.646	0.475	0.496	0.498	0.489	±0.013
48	0.405	0.396	0.403	0.401	±0.005	144	149	175	156	±16.64	0.494	0.518	0.514	0.512	±0.013
56	0.302	0.316	0.299	0.306	±0.009	124	127	145	132	±11.35	0.526	0.517	0.534	0.525	±0.009
64	0.228	0.223	0.216	0.222	±0.006	119	107	125	117	±9.165	0.537	0.532	0.542	0.536	±0.005
72	0.154	0.137	0.156	0.149	±0.010	098	102	094	098	±4.000	0.547	0.532	0.548	0.558	±0.009
80	0.110	0.110	0.123	0.114	±0.007	088	096	086	090	±5.292	0.558	0.532	0.547	0.545	±0.013

Avg – Average for all trials
SD- Standard deviation for all trials

Table D 3. Trials for 6.0 % v/v ethanol shock treatment in MSM production medium

Time (h)	TF yield (μmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.075	0.070	0.074	0.073	±0.002	065	059	065	063	±3.464	0.000	0.000	0.000	0.000	±0.000
8	0.142	0.146	0.145	0.170	±0.002	075	070	071	072	±2.646	0.128	0.134	0.145	0.135	±0.009
16	0.274	0.250	0.242	0.331	±0.016	110	117	118	115	±4.359	0.262	0.268	0.335	0.288	±0.041
24	0.344	0.362	0.371	0.462	±0.014	143	137	140	140	±3.000	0.334	0.358	0.458	0.376	±0.066
32	0.356	0.341	0.353	0.487	±0.008	135	141	139	138	±3.055	0.432	0.412	0.465	0.435	±0.027
40	0.343	0.325	0.332	0.470	±0.009	128	149	129	135	±11.84	0.475	0.456	0.474	0.468	±0.011
48	0.256	0.273	0.283	0.401	±0.014	112	121	112	115	±5.196	0.477	0.492	0.486	0.485	±0.008
56	0.202	0.215	0.219	0.306	±0.009	102	115	104	107	±7.000	0.476	0.484	0.481	0.480	±0.004
64	0.149	0.146	0.161	0.222	±0.008	094	104	096	098	±5.292	0.482	0.477	0.472	0.476	±0.005
72	0.124	0.117	0.104	0.149	±0.010	088	095	093	092	±3.606	0.460	0.466	0.482	0.465	±0.010
80	0.085	0.098	0.104	0.114	±0.010	082	104	085	090	±11.93	0.445	0.448	0.454	0.445	±0.005

Avg – Average for all trials
SD- Standard deviation for all trials

Table D 4. Trials for 7.5 % v/v ethanol shock treatment in MSM production medium

Time (h)	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.069	0.072	0.076	0.072	±0.003	067	058	061	062	±4.583	0.000	0.000	0.000	0.000	±0.000
8	0.137	0.143	0.126	0.134	±0.009	066	065	061	064	±2.646	0.045	0.034	0.037	0.039	±0.006
16	0.173	0.171	0.170	0.172	±0.002	082	071	075	076	±5.568	0.092	0.088	0.081	0.088	±0.006
24	0.254	0.257	0.276	0.262	±0.012	100	108	110	106	±5.292	0.142	0.132	0.140	0.145	±0.005
32	0.247	0.262	0.264	0.258	±0.009	100	108	110	106	±5.292	0.178	0.185	0.196	0.186	±0.009
40	0.234	0.238	0.236	0.236	±0.002	098	095	102	098	±3.512	0.216	0.214	0.227	0.219	±0.007
48	0.191	0.203	0.210	0.201	±0.010	088	094	089	090	±3.215	0.248	0.236	0.245	0.237	±0.006
56	0.123	0.134	0.102	0.119	±0.016	075	074	076	075	±1.000	0.256	0.242	0.251	0.248	±0.007
64	0.081	0.080	0.065	0.075	±0.009	066	069	057	064	±6.245	0.252	0.238	0.244	0.243	±0.007
72	0.049	0.056	0.058	0.054	±0.005	054	060	051	055	±4.583	0.227	0.241	0.236	0.232	±0.007
80	0.044	0.031	0.048	0.042	±0.009	037	045	044	042	±4.359	0.219	0.235	0.225	0.226	±0.008

Avg – Average for all trials
SD- Standard deviation for all trials

Table D 5. Trials for 3.0 % v/v methanol shock treatment in MSM production medium.

Time (h)	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.073	0.076	0.074	0.074	±0.001	069	062	067	066	±3.606	0.000	0.000	0.000	0.000	±0.000
8	0.271	0.274	0.268	0.271	±0.003	135	133	146	138	±7.000	0.052	0.062	0.055	0.056	±0.005
16	0.432	0.439	0.427	0.433	±0.006	193	202	199	198	±4.583	0.135	0.125	0.118	0.126	±0.009
24	0.501	0.512	0.527	0.513	±0.013	208	209	213	210	±2.646	0.184	0.176	0.182	0.175	±0.004
32	0.574	0.570	0.571	0.572	±0.002	217	225	218	220	±4.359	0.245	0.211	0.23	0.21	±0.017
40	0.591	0.603	0.591	0.595	±0.007	228	231	216	225	±7.937	0.286	0.248	0.275	0.263	±0.020
48	0.556	0.560	0.582	0.564	±0.014	215	217	222	218	±3.606	0.315	0.275	0.294	0.288	±0.020
56	0.473	0.507	0.477	0.485	±0.018	211	192	188	196	±12.288	0.336	0.308	0.318	0.312	±0.014
64	0.428	0.400	0.405	0.412	±0.015	184	188	192	172	±4.000	0.348	0.322	0.328	0.332	±0.014
72	0.347	0.353	0.359	0.352	±0.006	181	176	180	150	±2.646	0.356	0.321	0.326	0.324	±0.019
80	0.334	0.291	0.268	0.298	±0.034	169	182	159	131	±11.533	0.352	0.322	0.321	0.331	±0.018

Avg – Average for all trials
SD- Standard deviation for all trials

Table D 6. Trials for 4.5 % v/v methanol shock treatment in MSM production medium

Time (h)	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.072	0.077	0.070	0.073	±0.001	064	062	066	064	±2.000	0.000	0.000	0.000	0.000	±0.000
8	0.222	0.229	0.192	0.215	±0.020	119	128	128	125	±5.196	0.015	0.021	0.012	0.016	±0.005
16	0.402	0.409	0.405	0.405	±0.004	193	195	206	198	±7.000	0.032	0.036	0.045	0.035	±0.007
24	0.506	0.490	0.500	0.499	±0.008	212	210	214	212	±2.000	0.076	0.071	0.079	0.072	±0.003
32	0.527	0.558	0.549	0.545	±0.016	223	216	209	216	±7.000	0.108	0.116	0.114	0.112	±0.004
40	0.517	0.542	0.525	0.528	±0.013	208	213	209	210	±2.646	0.134	0.142	0.138	0.135	±0.004
48	0.500	0.499	0.495	0.498	±0.002	193	196	202	197	±4.583	0.156	0.153	0.16	0.156	±0.004
56	0.427	0.440	0.430	0.433	±0.007	188	181	186	185	±3.606	0.171	0.163	0.165	0.164	±0.004
64	0.374	0.366	0.360	0.366	±0.007	162	180	156	166	±12.490	0.172	0.168	0.166	0.166	±0.003
72	0.298	0.319	0.314	0.310	±0.011	132	155	139	142	±11.790	0.168	0.176	0.169	0.171	±0.004
80	0.259	0.289	0.276	0.275	±0.015	132	129	125	128	±3.512	0.166	0.172	0.160	0.174	±0.006

Avg – Average for all trials
SD- Standard deviation for all trials

Table D 7. Trials for 6.0 % v/v methanol shock treatment in MSM production medium.

Time (h)	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.077	0.078	0.072	0.075	±0.003	077	070	063	68	±7.000	0.000	0.000	0.000	0.000	±0.000
8	0.180	0.174	0.172	0.175	±0.004	117	112	128	119	±8.185	0.074	0.048	0.088	0.070	±0.020
16	0.330	0.342	0.333	0.335	±0.006	180	172	175	175	±4.041	0.224	0.177	0.217	0.206	±0.025
24	0.432	0.430	0.429	0.431	±0.001	188	205	198	197	±8.544	0.288	0.295	0.312	0.298	±0.012
32	0.477	0.490	0.493	0.487	±0.008	197	207	192	198	±7.638	0.369	0.338	0.365	0.356	±0.017
40	0.459	0.470	0.467	0.465	±0.006	185	212	180	192	±17.214	0.392	0.387	0.402	0.388	±0.008
48	0.423	0.415	0.430	0.422	±0.008	175	167	175	172	±4.619	0.417	0.395	0.435	0.413	±0.020
56	0.360	0.351	0.333	0.348	±0.013	150	156	160	155	±5.033	0.415	0.398	0.443	0.415	±0.023
64	0.288	0.282	0.287	0.286	±0.003	136	130	139	135	±4.583	0.426	0.415	0.445	0.424	±0.015
72	0.247	0.251	0.239	0.246	±0.006	117	119	124	120	±3.606	0.428	0.418	0.442	0.426	±0.012
80	0.226	0.220	0.224	0.223	±0.003	116	108	112	112	±4.000	0.415	0.422	0.438	0.435	±0.012

Avg – Average for all trials
SD- Standard deviation for all trials

Table D 8. Trials for 7.5 % v/v methanol shock treatment i n MSM production medium

Time (h)	TF yield (μmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.070	0.071	0.0076	0.0072	±0.003	630	68	061	064	±3.606	0.000	0.000	0.000	0.000	±0.000
8	0.152	0.150	0.131	0.144	±0.011	88	104	096	096	±8.000	0.096	0.086	0.098	0.094	±0.006
16	0.234	0.217	0.257	0.235	±0.020	119	132	133	128	±7.810	0.18	0.174	0.175	0.176	±0.003
24	0.314	0.329	0.308	0.317	±0.011	146	155	158	152	±6.245	0.252	0.244	0.258	0.252	±0.007
32	0.352	0.348	0.343	0.348	±0.004	153	167	160	160	±7.000	0.289	0.284	0.276	0.283	±0.007
40	0.381	0.363	0.327	0.352	±0.028	156	165	162	161	±4.583	0.314	0.316	0.308	0.313	±0.004
48	0.281	0.283	0.291	0.285	±0.005	135	139	140	138	±2.646	0.346	0.338	0.335	0.341	±0.006
56	0.211	0.217	0.234	0.221	±0.012	118	109	118	115	±5.196	0.376	0.377	0.364	0.372	±0.007
64	0.180	0.172	0.176	0.176	±0.004	098	097	099	098	±1.000	0.388	0.388	0.397	0.394	±0.005
72	0.133	0.149	0.134	0.139	±0.009	087	084	090	087	±3.000	0.402	0.392	0.41	0.399	±0.009
80	0.095	0.106	0.085	0.095	±0.011	068	070	078	072	±5.292	0.412	0.395	0.429	0.412	±0.017

Avg – Average for all trials
SD- Standard deviation for all trials

Table D 9. The activity of *S.venezuelae* as measured by specific TF yield for all alcohol shock temperatures.

Time (h)	Ethanol				Methanol			
	3 % v/v	4.5 % v/v	6 % v/v	7.5 % v/v	3 % v/v	4.5 % v/v	6 % v/v	7.5 % v/v
0	1.16×10^{-9}	1.15×10^{-9}	1.16×10^{-9}	1.16×10^{-9}	1.12×10^{-9}	1.14×10^{-9}	1.10×10^{-9}	1.13×10^{-9}
8	2.35×10^{-9}	2.13×10^{-9}	1.94×10^{-9}	2.09×10^{-9}	1.96×10^{-9}	1.72×10^{-9}	1.47×10^{-9}	1.50×10^{-9}
16	2.70×10^{-9}	2.46×10^{-9}	2.22×10^{-9}	2.26×10^{-9}	2.19×10^{-9}	2.05×10^{-9}	1.91×10^{-9}	1.84×10^{-9}
24	3.00×10^{-9}	2.75×10^{-9}	2.56×10^{-9}	2.47×10^{-9}	2.44×10^{-9}	2.35×10^{-9}	2.19×10^{-9}	2.09×10^{-9}
32	3.03×10^{-9}	2.75×10^{-9}	2.54×10^{-9}	2.43×10^{-9}	2.60×10^{-9}	2.52×10^{-9}	2.46×10^{-9}	2.18×10^{-9}
40	2.88×10^{-9}	2.69×10^{-9}	2.47×10^{-9}	2.41×10^{-9}	2.64×10^{-9}	2.51×10^{-9}	2.42×10^{-9}	2.19×10^{-9}
48	2.69×10^{-9}	2.57×10^{-9}	2.35×10^{-9}	2.23×10^{-9}	2.59×10^{-9}	2.53×10^{-9}	2.45×10^{-9}	2.07×10^{-9}
56	2.35×10^{-9}	2.32×10^{-9}	1.98×10^{-9}	1.59×10^{-9}	2.47×10^{-9}	2.34×10^{-9}	2.25×10^{-9}	1.92×10^{-9}
64	1.84×10^{-9}	1.90×10^{-9}	1.53×10^{-9}	1.17×10^{-9}	2.40×10^{-9}	2.20×10^{-9}	2.12×10^{-9}	1.80×10^{-9}
72	1.45×10^{-9}	1.52×10^{-9}	1.25×10^{-9}	0.98×10^{-9}	2.35×10^{-9}	2.18×10^{-9}	2.05×10^{-9}	1.60×10^{-9}
80	1.23×10^{-9}	1.27×10^{-9}	1.06×10^{-9}	0.95×10^{-9}	2.27×10^{-9}	2.15×10^{-9}	1.99×10^{-9}	1.32×10^{-9}

Appendix E. Multiple Ethanol Shock treatment (3 % v/v) on Streptomyces venezeuale at [0 h, (0, 3) h and 0, 3, 6) h] to initiate jadomycin B production in MSM production media.

Table E 1. Trials for 3.0 % v/v ethanol shock treatment at (0) h in MSM production medium.

Time	TF Yield (μmol)					CFU/mL x 10^6					OD 526 nm (AU)				
(h)	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.061	0.065	0.064	0.063	±0.002	054	056	058	056	±2.000	0.000	0.000	0.000	0.000	±0.000
10	0.226	0.223	0.225	0.225	±0.002	082	076	090	083	±7.024	0.114	0.120	0.112	0.115	±0.004
20	0.379	0.390	0.391	0.386	±0.006	134	141	140	138	±3.786	0.306	0.294	0.284	0.295	±0.011
30	0.466	0.460	0.462	0.463	±0.003	152	155	157	155	±2.517	0.368	0.392	0.355	0.372	±0.019
40	0.415	0.418	0.417	0.417	±0.002	145	148	141	145	±3.512	0.392	0.458	0.425	0.425	±0.033
50	0.311	0.313	0.321	0.315	±0.005	123	121	119	121	±2.000	0.465	0.474	0.466	0.468	±0.005
60	0.225	0.220	0.221	0.222	±0.003	112	102	098	104	±7.211	0.480	0.486	0.492	0.486	±0.006
70	0.143	0.146	0.137	0.142	±0.005	094	096	097	096	±1.528	0.492	0.495	0.490	0.492	±0.003
80	0.089	0.092	0.102	0.094	±0.007	078	077	079	078	±1.000	0.492	0.478	0.465	0.478	±0.014

Avg – Average for all trials
SD- Standard deviation for all trials

Table E 2. Trials for 3.0 % v/v ethanol shock treatment at (0, 3) h in MSM production medium.

Time	TF Yield (µmol)					CFU/mL x 10^6					OD 526 nm (AU)				
(h)	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0.062	0.068	0.065	0.065		±0.003	044	045	049	048	±2.646	0.000	0.000	0.000	0.000	±0.000
10	0.138	0.141	0.150	0.143	±0.006	072	076	073	074	±2.082	0.010	0.008	0.004	0.007	±0.003
20	0.216	0.215	0.222	0.218	±0.004	100	089	098	096	±5.859	0.048	0.055	0.039	0.047	±0.008
30	0.255	0.257	0.259	0.257	±0.002	112	125	110	116	±8.145	0.196	0.187	0.199	0.194	±0.006
40	0.246	0.249	0.252	0.249	±0.003	105	118	114	112	±6.658	0.261	0.272	0.221	0.251	±0.027
50	0.176	0.181	0.169	0.175	±0.006	073	089	079	080	±8.083	0.285	0.297	0.284	0.288	±0.007
60	0.107	0.105	0.102	0.105	±0.003	048	058	061	056	±6.807	0.267	0.279	0.273	0.273	±0.006
70	0.055	0.046	0.058	0.053	±0.006	048	046	042	045	±3.055	0.234	0.226	0.254	0.238	±0.014
80	0.041	0.039	0.012	0.031	±0.016	031	038	040	036	±4.726	0.217	0.236	0.221	0.224	±0.010

Avg – Average for all trials
SD- Standard deviation for all trials

Table E 3. Trials for 3.0 % v/v ethanol shock treatment at (0, 3 and 6) h in MSM production medium.

Time	TF Yield (µmol)					CFU/mL x 10^6					OD 526 nm (AU)				
(h)	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.060	0.062	0.065	0.062	±0.002	052	056	058	055	±3.06	0.000	0.000	0.000	0.000	±0.000
10	0.084	0.088	0.086	0.086	±0.002	052	049	085	062	±19.7	0.000	0.000	0.000	0.007	±0.000
20	0.200	0.209	0.197	0.202	±0.006	104	118	109	110	±7.09	0.004	0.002	0.002	0.047	±0.001
30	0.209	0.224	0.212	0.215	±0.008	112	110	115	112	±2.52	0.006	0.004	0.006	0.109	±0.001
40	0.135	0.127	0.132	0.131	±0.004	085	088	091	088	±3.00	0.022	0.016	0.018	0.121	±0.003
50	0.080	0.079	0.078	0.079	±0.001	053	056	055	055	±1.53	0.025	0.019	0.019	0.095	±0.003
60	0.058	0.057	0.047	0.054	±0.006	036	041	042	040	±3.21	0.027	0.021	0.023	0.090	±0.003
70	0.035	0.033	0.026	0.031	±0.004	034	038	033	035	±2.65	0.026	0.024	0.025	0.083	±0.001
80	0.015	0.005	0.014	0.012	±0.006	021	028	017	022	±5.57	0.022	0.018	0.019	0.081	±0.002

Avg – Average for all trials
SD- Standard deviation for all trials

Appendix F. ***Ethanol Shock treatment (3, 4.5 amd 6 % v/v) on Streptomyces venezeuale in MSM production media containing varying nitrogen (L-isoleucine) concentration (45, 60 and 75 mM) to initiate jadomycin B production in MSM production media.***

Table F 1. Trials for 3.0 % v/v ethanol shock treatment with 45 mM nitrogen concentration in MSM production medium.

Time (h)	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.059	0.057	0.05	0.054	±0.005	036	038	040	038	±2.000	0.000	0.000	0.000	0.000	±0.000
8	0.125	0.138	0.13	0.131	±0.007	069	065	070	068	±2.646	0.025	0.028	0.016	0.022	±0.006
16	0.291	0.282	0.29	0.287	±0.005	112	110	108	110	±2.000	0.082	0.088	0.086	0.084	±0.003
24	0.397	0.386	0.395	0.392	±0.006	124	128	132	128	±4.000	0.184	0.196	0.202	0.192	±0.009
32	0.399	0.396	0.394	0.396	±0.003	129	133	134	132	±2.646	0.248	0.262	0.248	0.255	±0.008
40	0.357	0.370	0.347	0.358	±0.012	121	125	129	125	±4.000	0.318	0.326	0.318	0.321	±0.005
48	0.274	0.288	0.276	0.279	±0.008	118	108	110	112	±5.292	0.342	0.334	0.356	0.344	±0.011
56	0.184	0.195	0.217	0.199	±0.017	092	091	088	090	±2.082	0.372	0.385	0.386	0.386	±0.008
64	0.172	0.141	0.152	0.155	±0.016	070	076	070	072	±3.464	0.402	0.398	0.416	0.407	±0.009
72	0.17	0.098	0.105	0.124	±0.040	052	058	058	056	±3.464	0.418	0.425	0.428	0.423	±0.005
80	0.082	0.088	0.077	0.082	±0.006	042	047	046	045	±2.646	0.436	0.432	0.458	0.440	±0.014

Avg – Average for all trials
SD- Standard deviation for all trials

Table F 2. Trials for 3.0 % v/v ethanol shock treatment with 60 mM nitrogen concentration in MSM production medium.

Time	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
(h)	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.056	0.052	0.051	0.053	±0.003	036	039	036	037	±1.732	0.000	0.000	0.000	0.000	±0.000
8	0.166	0.17	0.174	0.170	±0.004	071	075	070	072	±2.646	0.086	0.084	0.092	0.087	±0.004
16	0.362	0.295	0.319	0.325	±0.034	121	118	128	122	±5.132	0.235	0.228	0.245	0.236	±0.009
24	0.386	0.437	0.475	0.432	±0.045	142	140	150	144	±5.292	0.345	0.316	0.346	0.336	±0.017
32	0.452	0.475	0.492	0.473	±0.020	152	158	160	156	±4.163	0.378	0.396	0.382	0.385	±0.009
40	0.41	0.415	0.421	0.415	±0.006	142	146	144	144	±2.000	0.398	0.435	0.442	0.425	±0.024
48	0.325	0.31	0.375	0.336	±0.034	130	130	115	125	±8.660	0.466	0.462	0.451	0.460	±0.008
56	0.234	0.277	0.252	0.254	±0.022	103	114	113	110	±6.083	0.476	0.474	0.477	0.476	±0.002
64	0.19	0.182	0.192	0.188	±0.005	103	108	095	102	±6.557	0.498	0.496	0.492	0.495	±0.003
72	0.124	0.128	0.12	0.124	±0.004	090	088	098	092	±5.292	0.487	0.483	0.498	0.489	±0.008
80	0.087	0.105	0.096	0.096	±0.009	160	171	173	078	±7.000	0.482	0.479	0.466	0.476	±0.009

Avg – Average for all trials
SD- Standard deviation for all trials

Table F 3. Trials for 3.0 % v/v ethanol shock treatment with 75 mM nitrogen concentration in MSM production medium.

Time	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
(h)	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.056	0.054	0.05	0.053	±0.003	75	68	73	38	±3.606	0.000	0.000	0.000	0.000	±0.000
8	0.19	0.188	0.183	0.186	±0.004	127	118	121	82	±4.583	0.032	0.045	0.050	0.042	±0.009
16	0.393	0.331	0.355	0.359	±0.031	132	135	138	134	±3.000	0.112	0.092	0.104	0.103	±0.010
24	0.435	0.485	0.499	0.473	±0.034	146	141	139	156	±3.606	0.262	0.248	0.252	0.254	±0.007
32	0.518	0.521	0.546	0.528	±0.015	156	155	157	172	±1.000	0.338	0.336	0.319	0.331	±0.010
40	0.467	0.486	0.48	0.478	±0.010	165	159	168	164	±4.583	0.412	0.390	0.395	0.399	±0.012
48	0.387	0.384	0.392	0.388	±0.004	155	162	169	144	±7.000	0.431	0.442	0.425	0.433	±0.009
56	0.285	0.295	0.284	0.286	±0.006	193	200	195	124	±3.606	0.475	0.480	0.462	0.472	±0.024
64	0.210	0.215	0.216	0.212	±0.003	144	157	149	116	±6.557	0.512	0.512	0.471	0.498	±0.038
72	0.152	0.162	0.158	0.157	±0.005	142	149	144	102	±3.606	0.520	0.546	0.472	0.513	±0.002
80	0.122	0.125	0.127	0.124	±0.004	127	150	143	85	±11.79	0.518	0.516	0.520	0.518	±0.011

Avg – Average for all trials
SD- Standard deviation for all trials

Table F 4. Trials for 4.5 % v/v ethanol shock treatment with 45 mM nitrogen concentration in MSM production medium.

Time	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
(h)	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.053	0.058	0.054	0.055	±0.003	030	029	037	032	±4.359	0.000	0.000	0.000	0.000	±0.000
8	0.145	0.130	0.151	0.142	±0.011	071	077	068	072	±4.583	0.162	0.170	0.168	0.167	±0.004
16	0.258	0.265	0.274	0.266	±0.008	125	130	105	120	±13.22	0.344	0.338	0.329	0.337	±0.008
24	0.390	0.403	0.371	0.388	±0.016	165	168	172	168	±3.512	0.425	0.435	0.426	0.429	±0.006
32	0.425	0.437	0.445	0.435	±0.010	163	165	170	166	±3.606	0.485	0.496	0.504	0.495	±0.010
40	0.353	0.390	0.383	0.375	±0.020	140	155	149	148	±7.550	0.506	0.514	0.518	0.513	±0.006
48	0.294	0.284	0.287	0.288	±0.005	128	126	125	126	±1.528	0.532	0.536	0.548	0.539	±0.008
56	0.222	0.226	0.239	0.229	±0.009	112	116	117	115	±2.646	0.546	0.532	0.528	0.535	±0.009
64	0.170	0.155	0.150	0.158	±0.011	090	095	100	095	±5.000	0.533	0.518	0.496	0.516	±0.019
72	0.106	0.122	0.132	0.120	±0.013	085	088	084	085	±2.082	0.502	0.498	0.512	0.504	±0.007
80	0.093	0.090	0.094	0.092	±0.002	070	077	075	074	±3.606	0.480	0.487	0.472	0.480	±0.008

Avg – Average for all trials
SD- Standard deviation for all trials

Table F 5. Trials for 4.5 % v/v ethanol shock treatment with 60 mM nitrogen concentration in MSM production medium.

Time	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
(h)	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.055	0.058	0.056	0.056	±0.001	030	031	032	031	±1.000	0.000	0.000	0.000	0.000	±0.000
8	0.178	0.153	0.156	0.162	±0.014	081	085	070	078	±7.767	0.158	0.143	0.169	0.157	±0.013
16	0.277	0.292	0.286	0.285	±0.007	135	116	127	126	±9.539	0.327	0.308	0.318	0.318	±0.010
24	0.404	0.394	0.391	0.396	±0.006	162	145	150	152	±8.737	0.412	0.386	0.366	0.388	±0.025
32	0.429	0.458	0.462	0.450	±0.018	168	166	170	168	±2.000	0.442	0.478	0.448	0.456	±0.021
40	0.432	0.434	0.401	0.422	±0.019	162	168	148	159	±10.26	0.475	0.496	0.498	0.490	±0.010
48	0.369	0.357	0.373	0.367	±0.008	140	148	146	144	±4.163	0.494	0.518	0.514	0.509	±0.013
56	0.266	0.279	0.271	0.272	±0.007	121	114	113	116	±4.359	0.526	0.517	0.534	0.526	±0.010
64	0.198	0.197	0.200	0.198	±0.006	090	098	098	095	±4.619	0.537	0.532	0.542	0.537	±0.005
72	0.138	0.144	0.144	0.142	±0.009	078	086	077	082	±4.933	0.547	0.532	0.548	0.542	±0.011
80	0.113	0.108	0.109	0.110	±0.022	075	082	077	078	±3.606	0.558	0.532	0.547	0.545	±0.010

Avg – Average for all trials
SD- Standard deviation for all trials

Table F 6. Trials for 4.5 % v/v ethanol shock treatment with 75 mM nitrogen concentration in MSM production medium.

Time	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
(h)	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.055	0.060	0.056	0.057	±0.003	029	035	032	032	±3.000	0.000	0.000	0.000	0.000	±0.000
8	0.151	0.186	0.187	0.175	±0.021	079	086	075	080	±5.568	0.168	0.160	0.158	0.162	±0.005
16	0.336	0.343	0.298	0.325	±0.025	141	125	137	134	±8.327	0.285	0.315	0.290	0.297	±0.016
24	0.452	0.457	0.461	0.456	±0.004	176	166	162	168	±7.211	0.382	0.378	0.366	0.375	±0.008
32	0.533	0.486	0.518	0.512	±0.024	177	181	184	180	±3.512	0.415	0.442	0.420	0.426	±0.014
40	0.444	0.511	0.534	0.496	±0.047	170	177	178	175	±4.359	0.476	0.485	0.455	0.472	±0.015
48	0.425	0.401	0.410	0.412	±0.012	158	155	155	156	±1.732	0.524	0.530	0.522	0.525	±0.004
56	0.335	0.321	0.327	0.328	±0.007	133	144	125	133	±9.539	0.536	0.535	0.568	0.546	±0.019
64	0.257	0.254	0.246	0.252	±0.006	110	118	124	117	±7.024	0.581	0.576	0.562	0.573	±0.010
72	0.194	0.177	0.185	0.185	±0.009	102	095	097	098	±3.606	0.616	0.598	0.623	0.612	±0.013
80	0.127	0.137	0.170	0.145	±0.022	091	088	092	090	±2.082	0.634	0.624	0.614	0.624	±0.010

Avg – Average for all trials
SD- Standard deviation for all trials

Table F 7. Trials for 6.0 % v/v ethanol shock treatment with 45 mM nitrogen concentration in MSM production medium.

Time (h)	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.039	0.041	0.046	0.042	±0.003	034	036	035	035	±1.000	0.000	0.000	0.000	0.000	±0.000
8	0.141	0.133	0.138	0.137	±0.011	079	075	080	078	±2.646	8.000	0.052	0.067	0.046	±0.011
16	0.204	0.228	0.223	0.218	±0.008	116	121	108	115	±6.557	16.000	0.192	0.188	0.186	±0.003
24	0.321	0.321	0.330	0.324	±0.016	126	128	136	130	±5.292	24.000	0.282	0.296	0.274	±0.011
32	0.347	0.355	0.371	0.358	±0.010	133	135	134	134	±1.000	32.000	0.350	0.372	0.364	±0.011
40	0.297	0.294	0.345	0.312	±0.020	119	124	120	121	±2.646	40.000	0.415	0.432	0.430	±0.009
48	0.215	0.218	0.208	0.214	±0.005	098	110	089	099	±10.53	48.000	0.472	0.440	0.458	±0.016
56	0.149	0.170	0.161	0.160	±0.009	082	079	080	082	±1.528	56.000	0.480	0.470	0.476	±0.005
64	0.104	0.109	0.104	0.106	±0.011	068	072	065	068	±3.512	64.000	0.482	0.472	0.478	±0.005
72	0.083	0.081	0.067	0.077	±0.013	053	058	063	058	±5.000	72.000	0.462	0.468	0.456	±0.006
80	0.060	0.055	0.056	0.057	±0.002	050	052	050	050	±1.155	80.000	0.454	0.446	0.436	±0.009

Avg – Average for all trials
SD- Standard deviation for all trials

Table F 8. Trials for 6.0 % v/v ethanol shock treatment with 60 mM nitrogen concentration in MSM production medium.

Time	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
(h)	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.044	0.046	0.043	0.044	±0.001	036	031	038	035	±3.606	0.000	0.000	0.000	0.000	±0.000
8	0.151	0.153	0.155	0.153	±0.014	081	085	080	082	±2.646	0.128	0.134	0.145	0.136	±0.009
16	0.266	0.266	0.286	0.272	±0.007	119	120	126	122	±3.786	0.262	0.268	0.335	0.288	±0.041
24	0.355	0.353	0.367	0.358	±0.006	135	136	144	138	±4.933	0.334	0.358	0.458	0.383	±0.066
32	0.393	0.392	0.410	0.398	±0.018	144	149	145	146	±2.646	0.432	0.412	0.465	0.436	±0.027
40	0.362	0.361	0.345	0.356	±0.019	118	138	125	127	±10.14	0.475	0.432	0.474	0.460	±0.025
48	0.259	0.282	0.283	0.275	±0.008	120	098	112	110	±11.13	0.477	0.492	0.486	0.485	±0.008
56	0.187	0.180	0.198	0.188	±0.007	088	090	086	088	±2.000	0.476	0.484	0.480	0.480	±0.004
64	0.140	0.134	0.126	0.133	±0.006	071	076	075	074	±2.646	0.482	0.477	0.472	0.477	±0.005
72	0.088	0.094	0.095	0.092	±0.009	072	059	055	062	±8.888	0.443	0.466	0.487	0.465	±0.022
80	0.058	0.079	0.079	0.072	±0.022	060	054	055	056	±3.215	0.435	0.448	0.454	0.446	±0.010

Avg – Average for all trials
SD- Standard deviation for all trials

Table F 9. Trials for 6.0 % v/v ethanol shock treatment with 75 mM nitrogen concentration in MSM production medium.

Time (h)	TF yield (µmol)					CFU/mL (x 10^6)					Jadomycin Production (AU 526nm)				
	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD	Trial 1	Trial 2	Trial 3	Avg	SD
0	0.044	0.046	0.049	0.046	±0.003	033	029	037	033	±4.000	0.000	0.000	0.000	0.000	±0.000
8	0.189	0.178	0.186	0.184	±0.021	092	086	091	089	±3.215	0.215	0.226	0.210	0.217	±0.008
16	0.308	0.315	0.324	0.316	±0.025	122	125	126	124	±2.082	0.362	0.349	0.368	0.360	±0.010
24	0.412	0.429	0.417	0.419	±0.004	150	156	138	148	±9.165	0.412	0.425	0.410	0.416	±0.008
32	0.448	0.465	0.453	0.455	±0.024	150	152	154	152	±2.000	0.462	0.478	0.486	0.475	±0.012
40	0.399	0.394	0.391	0.395	±0.047	135	138	135	136	±1.732	0.506	0.518	0.514	0.513	±0.006
48	0.312	0.310	0.304	0.309	±0.012	112	134	108	118	±14.00	0.538	0.535	0.537	0.537	±0.002
56	0.226	0.239	0.236	0.234	±0.007	096	102	118	105	±11.37	0.544	0.537	0.542	0.541	±0.004
64	0.173	0.180	0.170	0.174	±0.006	094	094	088	092	±3.464	0.542	0.538	0.540	0.540	±0.002
72	0.133	0.132	0.134	0.133	±0.009	082	084	090	085	±4.163	0.570	0.552	0.552	0.558	±0.010
80	0.113	0.108	0.098	0.106	±0.022	077	078	085	080	±4.359	0.555	0.572	0.568	0.565	±0.009

Avg – Average for all trials
SD- Standard deviation for all trials

Table F 10. Specific TF for ethanol induced nutrient shock treatment in MSM production media.

Time (h)	3.0 % v/v ethanol			4.5 % v/v ethanol			6.0 % v/v ethanol		
	45 mM	60 mM	75 mM	45 mM	60 mM	75 mM	45 mM	60 mM	75 mM
0	1.42×10^{-9}	1.43×10^{-9}	1.39×10^{-9}	1.42×10^{-9}	1.39×10^{-9}	1.41×10^{-9}	1.35×10^{-9}	1.38×10^{-9}	1.39×10^{-9}
8	1.92×10^{-9}	2.26×10^{-9}	2.34×10^{-9}	1.97×10^{-9}	2.02×10^{-9}	2.19×10^{-9}	1.76×10^{-9}	1.87×10^{-9}	2.07×10^{-9}
16	2.46×10^{-9}	2.66×10^{-9}	2.77×10^{-9}	2.22×10^{-9}	2.32×10^{-9}	2.43×10^{-9}	2.10×10^{-9}	2.232×10^{-9}	2.54×10^{-9}
24	2.77×10^{-9}	3.03×10^{-9}	3.06×10^{-9}	2.31×10^{-9}	2.61×10^{-9}	2.71×10^{-9}	2.49×10^{-9}	2.59×10^{-9}	2.83×10^{-9}
32	2.68×10^{-9}	2.93×10^{-9}	2.99×10^{-9}	2.62×10^{-9}	2.68×10^{-9}	2.84×10^{-9}	2.67×10^{-9}	2.80×10^{-9}	2.99×10^{-9}
40	2.52×10^{-9}	2.8×10^{-9}	2.88×10^{-9}	2.53×10^{-9}	2.65×10^{-9}	2.83×10^{-9}	2.58×10^{-9}	2.79×10^{-9}	2.90×10^{-9}
48	2.21×10^{-9}	2.58×10^{-9}	2.69×10^{-9}	2.29×10^{-9}	2.55×10^{-9}	2.64×10^{-9}	2.16×10^{-9}	2.50×10^{-9}	2.61×10^{-9}
56	1.93×10^{-9}	2.3×10^{-9}	2.43×10^{-9}	1.96×10^{-9}	2.34×10^{-9}	2.48×10^{-9}	1.95×10^{-9}	2.14×10^{-9}	2.23×10^{-9}
64	1.54×10^{-9}	1.84×10^{-9}	2.10×10^{-9}	1.66×10^{-9}	2.08×10^{-9}	2.15×10^{-9}	1.54×10^{-9}	1.78×10^{-9}	1.89×10^{-9}
72	1.34×10^{-9}	1.53×10^{-9}	1.91×10^{-9}	1.41×10^{-9}	1.73×10^{-9}	1.89×10^{-9}	1.31×10^{-9}	1.48×10^{-9}	1.56×10^{-9}
80	1.23×10^{-9}	1.45×10^{-9}	1.82×10^{-9}	1.24×10^{-9}	1.41×10^{-9}	1.62×10^{-9}	1.12×10^{-9}	1.29×10^{-9}	1.33×10^{-9}