

Changes in Cell Structure, Morphology and Activity of *Streptomyces venezuelae* during the Growth, Shocking and Jadomycin Production Stages

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Abstract

Streptomyces venezuelae have the ability to synthesize a group of novel benzoxazolo-phenanthridine antibiotics called jadomycin. The aim of the study was to investigate the changes in activity, cell structure and morphology of *Streptomyces venezuelae* while subjected to different environmental conditions during the growth, ethanol shocking, and jadomycin production stages. The activity of *S. venezuelae* was measured using the triphenyl tetrazolium chloride (TTC) technique while the microbial population was measured using the optical density and plate count techniques. Samples from each stage were viewed under the scanning electron microscope (SEM) and transmission electron microscope (TEM). The specific TF yield was calculated by dividing the TF yield by the number of cells. The specific TF yield remained constant at 2.44×10^{-8} ($\mu\text{mol}/\text{CFU}$) during the growth period in nutrient rich medium and decreased to 0.25×10^{-8} $\mu\text{mol}/\text{CFU}$ and 0.28×10^{-8} $\mu\text{mol}/\text{CFU}$ during the acclimatization to the nutrient deprived-amino acid rich production medium and after shocking and then increased to 3.67×10^{-8} $\mu\text{mol}/\text{CFU}$ during jadomycin production. The ethanol shock did not cause 100% of the cells to change their morphology. Remarkable changes were observed in the morphology and structure (cell diameter, vacuoles present and septation/sporulation) of *S. venezuelae* during the four consecutive stages (growth, acclimatization, shocking and jadomycin production). The elemental analysis provided information for verifying jadomycin B purity (97.86%). However, it was not possible to detect jadomycin B within cells. It is probable that jadomycin was produced outside the cells by extracellular enzymes as opposed to intracellularly by the uptake of isoleucine and glucose. The white pellets (cells), obtained by centrifugation of production medium, supports the idea that jadomycin B is produced outside the cells as jadomycin is highly coloured compound. It is also possible that the cells were highly efficient at excreting the secondary metabolites. These hypotheses need further investigation.

Keywords: Dehydrogenase activity; Triphenyl tetrazolium chloride (TTC); Jadomycin; Scanning electron microscopy (SEM); Transmission electron microscopy (TEM); *Streptomyces venezuelae*; Structure; Morphology

Introduction

The actinomycete *Streptomyces venezuelae* are capable of producing secondary metabolites such as jadomycin and chloramphenicol (Figure 1) [1]. Jadomyins have demonstrated antibacterial, antitumor, antifungal, enzyme inhibitory as well as cytotoxic properties to cancer cells [2]. All these properties make jadomyins promising novel antibiotic and cancer treatment drugs [3]. The production of jadomyins takes place in a nutrient-deprived amino acid rich medium. This can be achieved after the exhaustion of carbon, nitrogen or phosphate from the growth medium and the addition of amino acids and is induced by environmental shock using ethanol or heat [4]. The formation of the cyclized product of jadomycin is due to the presence of amino acids in the culture medium which has a biosynthetic aldehyde precursor that generates a reactive aldimine to form jadomycin [5]. The production yield of jadomycin has been extensively linked to the mass of vegetative cells of *Streptomyces venezuelae* that is transferred from the growth medium to production medium [6].

Dehydrogenase activity measurement can be used for the determination of bacterial growth and metabolism [7]. The test is based on the principle that the dehydrogenase enzymes are produced by all living cells and the extent to which the enzymes oxidize organic matter can be related to the number of living cells and their activities [8]. The dehydrogenase enzymes transport electrons and a hydrogen atom from an oxidized matrix to an electron acceptor [9, 10], and the dehydrogenase activity can be measured using the colourless triphenyl

tetrazolium chloride (TTC) salt as a hydrogen acceptor which turns to a red triphenyl formazan (TF) dye after the oxidization of organic matter [11,12] as shown in Figure 2.

The purpose of this study was to evaluate the activity, morphology and structure of *S. venezuelae* during the growth, shocking and jadomycin production stages and determine the purity of jadomycin produced.

Materials and Method

Chemicals and reagents

Triphenyl tetrazolium chloride (TTC) salt was used to prepare TTC-glucose solution for measuring the dehydrogenase activity of *S. venezuelae* cells. Tris(hydroxymethyl)aminomethane and NaOH were used to prepare the Tris buffer solution to control the pH of the samples. Triphenyl formazan (TF) was used to establish standard curves for TF concentration versus absorbance and TF yield versus cell number,

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Component	Chemical Formula	Quantity (per L distilled water)	Stock Solution Preparation (g/L distilled water)
MOPS (Organic buffer)	C ₇ H ₁₅ NO ₄ S	1.9 g	
Salt Solution			
Sodium Chloride	NaCl	9.0 mL	10.0
Calcium Chloride	CaCl ₂	9.0 mL	10.0
Magnesium Sulfate	MgSO ₄	0.4 g	
Ferrous Sulfate Solution (0.2%)	FeSO ₄ ·7H ₂ O	4.5 mL	2.0
Trace Mineral Solution			
Zinc Sulfate	ZnSO ₄ ·7H ₂ O	4.5 mL	0.880
Cupric Sulfate	CuSO ₄ ·5H ₂ O	4.5 mL	0.039
Manganese Sulfate	MnSO ₄ ·4H ₂ O	4.5 mL	0.0061
Boric Acid	H ₃ BO ₃	4.5 mL	0.0057
Ammonium Molybdate	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	4.5 mL	0.0037
Isoleucine (Amino acids)	C ₆ H ₁₃ NO ₂	7.8 g	
Glucose	C ₆ H ₁₂ O ₆	20.0 mL	5.94
Sodium Phosphate Monobasic Solution	NaH ₂ PO ₄ ·H ₂ O	5.5 mL	1.24

Table 2: Production medium components.

aliquots from salt, ferrous sulphate, trace mineral, glucose, and phosphate stock solutions.

The stock of the salt solution was prepared by adding 5 g NaCl and 5 g CaCl₂ into a 500 mL vessel and bringing the total volume to 500 mL with distilled water. The stock of ferrous sulfate solution was prepared by adding 1 g FeSO₄·7H₂O into a 500 mL vessel and bringing the total volume to 500 mL. The stock of trace mineral solution was prepared by adding 880 mg ZnSO₄·7H₂O, 39 mg CuSO₄·5H₂O, 6.1 mg MnSO₄·4H₂O, 5.7 mg H₃BO₃, and 3.7 mg (NH₄)₆Mo₇O₂₄·4H₂O into a 1 L vessel and bringing the total volume to 1 L with distilled water. A 30 mM glucose solution was prepared by adding 5.94 g glucose into a 50 mL vessel and bringing the total volume to 20 mL with distilled water. The phosphate stock solution was prepared by adding 1.24 g NaH₂PO₄·H₂O into a 1 L vessel and bringing the total volume into 1 L with distilled water.

An initial solution was prepared by adding 0.4 g MgSO₄, 1.9 g MOPS, 7.8 g isoleucine, 9 mL salt solution (1% w/v NaCl; 1% w/v CaCl₂), 4.5 mL 0.2% FeSO₄·7H₂O, and 4.5 mL trace mineral solution into a 1 L vessel. The total volume was brought to 1 L with distilled water. This solution and the glucose and phosphate solutions were autoclaved separately to avoid any precipitation. All solutions were autoclaved (SterileMax, Harvey/Barnstead International of Thermo Fisher Scientific, Ottawa, Ontario, Canada) on the liquid setting (121°C, 20 Pa) for 15 minutes. Upon cooling to room temperature, 5.5 mL phosphate stock solution and 20 mL of glucose solution were added aseptically to the vessel containing the other media components of the production medium. Aliquots of 175 mL of this medium were aseptically added to previously sterilized 250 mL flasks, covered with foam and aluminum foil and stored in the refrigerator until needed.

Triphenyl formazan standard curve

To determine the absorbance measured at 484 nm (AU₄₈₄) corresponding to the triphenyl formazan (TF) concentration, a standard curve was developed. A TF stock solution of 0.2 μmol/mL was prepared by dissolving 0.03 g triphenyl formazan in 500 mL methanol. From the stock solution, a set of 11 solutions were prepared with triphenyl concentrations of 0.004 μmol/mL, 0.01 μmol/mL, 0.02 μmol/mL, 0.03 μmol/mL, 0.04 μmol/mL, 0.05 μmol/mL, the stock solution, a set of 11 solutions were prepared with triphenyl concentrations of 0.004 μmol/mL, 0.06 μmol/mL, 0.07 μmol/mL, 0.08 μmol/mL, 0.09 μmol/mL, and 0.1 μmol/mL. The absorbance of the 11 solutions were measured at a wavelength of 484 nm. The spectrophotometer (Genesys

20, Thermo Scientific, Mississauga, Ontario, Canada) was zeroed with a blank sample of methanol. The absorbance readings were plotted against the prepared triphenyl formazan concentrations (μmol/mL) as shown in Figure 4. The following linear relationship between the absorbance (AU₄₈₄) and the TF was obtained (R² = 0.98).

$$AU_{484} = 10.574 TF \quad (1)$$

where: AU₄₈₄ is the absorbance reading at 484 nm TF is the concentration of triphenyl formazan (μmol/mL extraction solvent)

Streptomyces venezuelae growth on MYM agar plates

The liquified MYM agar (15 mL) was aseptically poured into individual petri plates and left to solidify. After the solidification of agar, a loopful of culture from the initial starter plate of *Streptomyces venezuelae* ISP5230 was streaked on each petri plate using a disposable inoculation loop. The inoculated plates were incubated at 30°C for 48 hours until visible colonies formed. The subcultured petri plates were preserved at 4°C in the refrigerator until used for inoculating the liquid MYM growth media.

Triphenyl formazan-CFU standard curve

In order to determine the TF yield corresponding to a given number of live cells, a standard curve was established. One of the 250 mL flask containing 175 mL autoclaved MYM broth was inoculated with *Streptomyces venezuelae* ISP5230 from MYM agar plates. The flask was incubated in an environmentally controlled shaker (25 Incubator Shaker, New Brunswick Scientific, Edison, New Jersey, USA) at 30°C and 250 rpm for 40 hours. Samples were taken at 1, 3, 10, 20, 25, 35 hours and the TTC and plate count test were performed on the samples. TF yield was plotted versus the number of CFU as shown in Figure 5.

Experimental protocol

Three of the 250 mL flasks containing 175 mL autoclaved MYM broth were inoculated with *S. venezuelae* ISP5230 from MYM agar plates and incubated in a controlled environmental shaker (25 Incubator Shaker, New Brunswick Scientific, Essdison, New Jersey, USA) at 30°C and 250 rpm. Samples were collected from the three flasks at 2, 12, 14, 21, 23, 38, 40, 42, 60 and 64 hours after incubation and the extent of cell growth was monitored over the period of 64 hours. The optical density was measured at 600 nm using a spectrophotometer

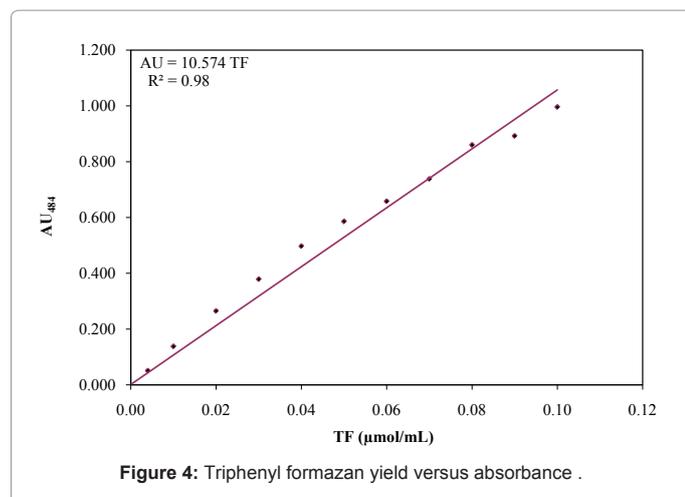


Figure 4: Triphenyl formazan yield versus absorbance .

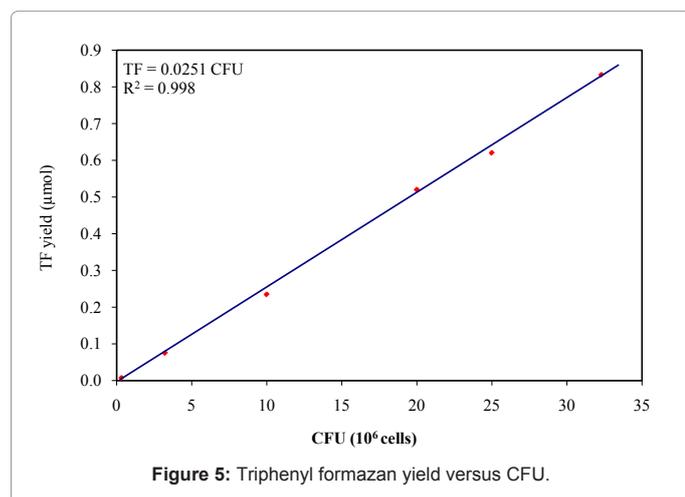


Figure 5: Triphenyl formazan yield versus CFU.

(Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada) and the CFU was determined using Equation 2. The dehydrogenase activity was also determined by measuring the absorbance at 484 nm (AU_{484}) and determining the TF yield using Equation 1. The samples collected after 40 hours of growth in the nutrient rich medium were also used for observation under the electron microscopes.

The remaining contents of the flask were used to provide cells for jadomycin B production. Three aliquots of 45 mL each were placed in three tubes and centrifuged (IEC CentraCL2, Thermo Electron Corporation, Mississauga, Ontario, Canada) for 10 minutes to separate cells from other media components. The supernatant was discarded and the pellet in each tube was re-suspended in 10 mL production medium. The resulting slurry from each tube was added to a 250 mL flask containing the 175 mL production medium. The flasks were incubated in an environmentally controlled shaker (25 Incubator Shaker, New Brunswick Scientific, Edison, New Jersey, USA) at 30°C and 250 rpm for 3 hours to allow the bacteria to acclimatize to the production medium and grow. Samples were collected following the 3 hour incubation period for plate count and TTC tests and observation under the electron microscopes. After 3 hour incubation, ethanol (100%) was added to the flasks (3% v/v) to shock the bacteria and start jadomycin B production. Samples were collected at 0, 8, 16, 24, 32, 48 and 72 hours after ethanol shock for plate count and observation under the electron microscopes.

Samples for jadomycin analysis were taken immediately after the ethanol shock and after 24 hours of jadomycin B production. The samples were viewed under SEM and TEM. Purified jadomycin was also viewed under SEM. The absorbance was also measured at the 526 nm (Au_{526}) as recommended by Jakeman et al. [6].

CFU determination

A series of dilutions were carried out for the determination of the number of CFU. A 1 mL aliquot of the original sample was added to an autoclaved test tube containing 9 mL of autoclaved distilled water. The test tube was capped and inverted several times to distribute the cells. An aliquot of 1 mL of this solution was added to a second autoclaved test tube containing 9 mL of autoclaved distilled water. This tube was capped and inverted to distribute the cells. This was carried out seven more times to a final dilution of 10^{-10} . For each of the six dilutions used (10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10}), 0.1 mL was added to a petri dish containing MYM agar in triplicate (given final plate dilutions of 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} , and 10^{-11}). The plates were sealed with parafilm, inverted, and incubated at 30°C in an environmentally controlled incubator (model number 2020, VWR International, Cornelius, Oregon, USA) for 24 hours. Following the incubation period, the plates were removed and the colonies were counted. The plates that had between 30-300 CFU present were used for calculating the CFU of the samples.

Dehydrogenase activity measurement

1 mL was pipetted from each sample into four test tubes. Tris buffer (2.5 mL) and TTC-glucose solution (1 mL) were added to the sample tubes (1 mL of distilled water was added to the control tube). The pH was adjusted to 6 using 1.0 N HCl and the test tubes were gently swirled to mix the content. The tubes were incubated in an environmentally controlled incubator (model number 2020, VWR International, Cornelius, Oregon, USA) at 30°C for 1 hour. The tubes were removed and centrifuged (IEC CentraCL2, Thermo Electron Corporation, Mississauga, Ontario, Canada) for 10 minutes to separate the cells from other medium components. TF extraction was carried out three times using 2.5 mL of methanol each time. All samples were vortexed (Thermolyne Maxi Mix, Thermolyne Corporation, Hampton, New Hampshire, USA) to disrupt cell walls and leach TF from within cells followed by centrifugation to separate the cells at the bottom. Supernatants from the three extractions were combined and the absorbance of the combined supernatants was measured at 484 nm (Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada).

Scanning electron microscope (SEM) analysis

Each sample was filtered using medium porosity filter paper (P5, Fisher Scientific, Ottawa, Ontario, Canada) and 0.8 μm filter paper (Cat. No. AAWP14250, Millipore, Billerica, MA, USA). The filter paper containing the cells was stored in a capped glass vial in the refrigerator at 4°C until needed for analysis. The filter paper was first dried on stubs and then coated with a gold/palladium mixture for 75 seconds to achieve a final thickness of 180 nm. The samples were viewed using a cold field emission scanning electron microscope (S-4700, Hitachi, Mississauga, Ontario, Canada) and elemental analysis was determined using an energy dispersive spectrometer (X-Sight, Oxford Instruments, Oxfordshire, UK). Cell sizes were estimated from micrographs using scale bars. Ten cell lengths and diameters were estimated and the average value taken.

Transmission electron microscopy (TEM) analysis

Aliquots of 2 mL of each sample were put into a 2.0 mL capped test

tubes and centrifuged in a microcentrifuge (Mini Centrifuge, Cat. No. 05-090-100, Fisher Scientific, Ottawa, Ontario, Canada) for 15 minutes. The supernatant was decanted and 1.0 mL of 2.5% glutaraldehyde was added to the tube. The pellet was re-suspended by gentle shaking and the tube was refrigerated at 4°C until needed for analysis. After a minimal fixation time of two hours in 2.5% glutaraldehyde, samples were rinsed for 10 minutes with 0.1 M sodium cacodylate buffer three times. Samples were then fixed for two hours in 1% sodium cacodylate buffer, after which they were rinsed with distilled water. Samples were left at 4°C overnight in 0.25% uranyl acetate. Samples were dehydrated with a graduated series of acetone (50% for 10 minutes, 50% for 10 minutes, 100% for 10 minutes, 70% for 10 minutes, 70% for 10 minutes, 95% for 10 minutes, 95% for 10 minutes, 100% for 10 minutes, 100% for 10 minutes and 100% for 10 minutes). Epon Araldite Resin was used to penetrate the samples in three stages: 3:1 ratio (dried 100% acetone: Resin) for three hours, 1:3 ratio overnight, and 100% Epon Araldite Resin for three hours. Samples were then embedded in 100% Epon Araldite Resin and left to cure in a 60°C oven for 48 hours. After hardening, thin sections were cut to approximately 100 nm thickness using a LKB Huxley Ultramicrotome (LKB Instruments, Inc., Rockville, Maryland, USA) with a diamond knife. On a 300 mesh copper grid, samples were stained for 10 minutes with 2% aqueous uranyl acetate, rinsed twice for five minutes with distilled water, exposed to lead citrate for four minutes, rinsed again with distilled water, and left to air dry. Samples were viewed with a transmission electron microscope (FEI Tecnai-12, Philips Electron Optics, Eindhoven, Netherlands) and images were obtained with a digital camera (MegaView II, Olympus Soft Imaging Solutions, Lakewood, Colorado, USA) Cell lengths and diameters were investigated using the TEM software. Between four and seven cells were measured and the observed lengths and diameters averaged.

Jadomycin purification and analysis

Samples were filtered through five layers of medium porosity filter paper (P5, Fisher Scientific, Ottawa, Ontario, Canada) and then through 0.8 µm filter paper (Cat. No. AAWP14250, Millipore, Billerica, Massachusetts, USA) to remove smaller residual media components. After filtration, the solution was passed through an isolute flash C18 chromatography column (Biotage, Charlottesville, Virginia, USA) after the column was first hydrated by rinsing it with distilled water. The components trapped in the column were removed through a series of methanol-water solutions increasing methanol concentration (10-100% methanol). The resulting orange filtrates from the various concentrations for each methanol-water concentrations were saved in separate flasks. The solutions were then dried in a round bottom flask using a rotary evaporator (R-200, Büchi, Flawil, Switzerland) with a CryoCool unit (Neslab Instruments Inc., Portsmouth, New Hampshire, USA), dry vacuum pump (Welch, Sheboygan, Wisconsin, USA) and heating water bath (B-490, Büchi, Flawil, Switzerland). The dried solution was re-suspended in 100% methanol and trapped on silica chromatography resin. The resin containing jadomycin B was packed in a column for the final stage of purification in a biotage (SP1, Biotage, Charlottesville, Virginia, USA). The purified jadomycin, in a methanol-water solution, was dried using the rotary evaporator and the residue re-suspended in a minimal volume of 100% methanol and stored at 0°C. Thin layer chromatography (TLC) was carried out to verify the purity of the jadomycin B. The purified jadomycin B sample was spotted on phase silica gel plates (Silicycle Inc., Quebec City, Quebec, Canada). The plates were run in DCM:MeOH:H₂O, (95.6:4.0:0.2). The powder was viewed under the scanning electron microscope (S-4700,

Hitachi, Mississauga, Ontario, Canada). An elemental analysis was also carried out on purified jadomycin powder.

Results

Morphological and structural changes

Streptomyces venezuelae were exposed to four different environments: (a) normal growth in MYM broth (b) acclimatization to nutrient-deprived, amino acid-rich production medium (c) ethanol shock in the production medium and (d) jadomycin B production in the production medium.

Growth in MYM broth

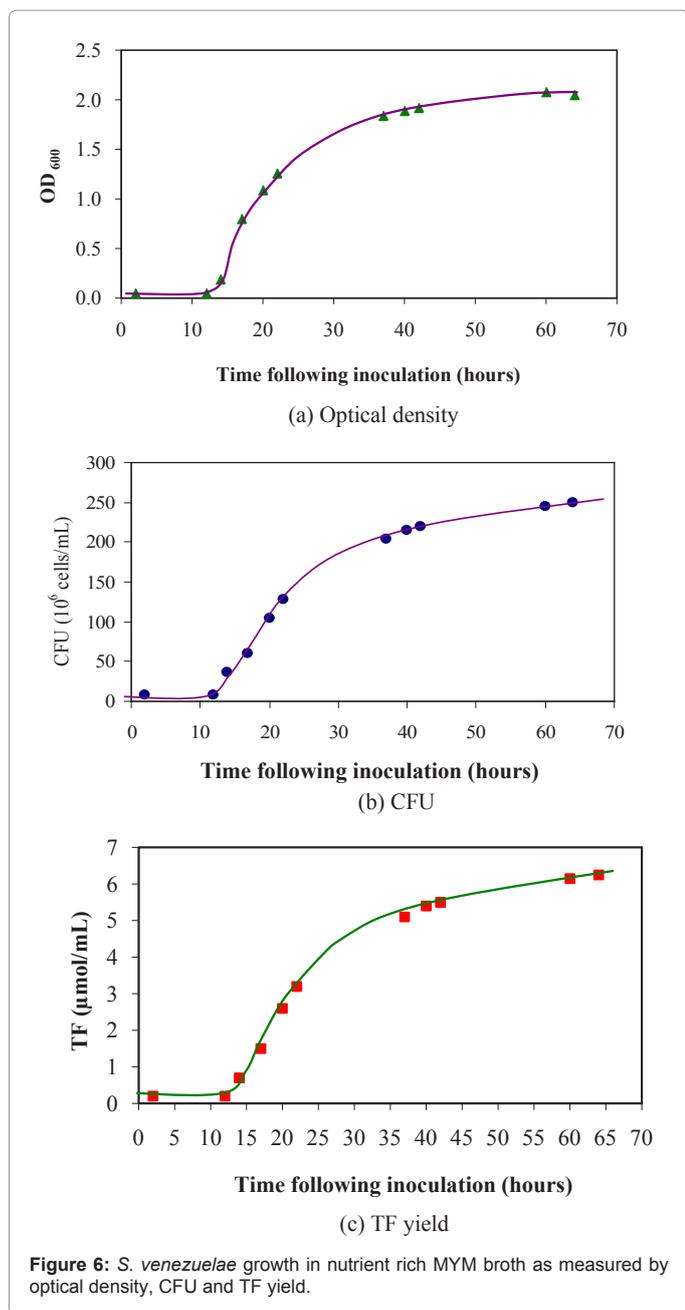
The growth of *S. venezuelae* was determined by: (a) measuring the optical density at 600nm (OD₆₀₀), (b) measuring the absorbance at 484nm (AU₄₈₄) and calculating the triphenyl formazan yield (TF) from Equation 1 and (c) determining the number of colony forming units (CFU) from Equation 2. The results are presented in Figure 6. An initial lag period of 12 hours was observed followed by an exponential growth period that ended after 60 hours from the start. During the lag period, the bacteria adjusted to the new environmental condition in the growth medium and produced the enzyme required for the utilization of the substrate in the media.

After 40 hours of growth in a nutrient-rich MYM broth, *S. venezuelae* reached an average cell concentration of 2.25 x 10⁸ CFU per mL of MYM broth. This corresponded to an optical density reading at 600 nm (OD₆₀₀) of 1.75 and a triphenyl formazan (TF) yield of 5.5 µmol. Therefore, the specific TF yield was 2.44 x 10⁻⁸ µmol/CFU.

The SEM image of cells in the sample taken after 40 hours of growth in the nutrient rich MYM broth showed a bed of filamentous hyphae (Figure 7a). The cells appeared fairly uniform (width between 0.5-1 µm). Some strands of hyphae appeared segmented into shorter rods (approximately 2.5 µm by 1 µm). Cross-sections of *S. venezuelae* hyphae are present in the TEM images (Figure 7b). Portions of vegetative hyphae that were alive and disintegrating were observed. A longitudinal cross-section of a vegetative hyphae cell (H) with a width that varied between 0.4-0.7 µm and a length of over 6 µm was seen. It comprised of an electron-dense cytoplasm enclosed by a plasma membrane (PM) and cell wall (W), with a few small globular electron-transparent vacuoles (Vac) present (below 100 nm in width) within the cytoplasm. The globular vacuoles appear much larger (up to 800 nm in width) and more numerous in the other living hyphae cells pictured in Figure 7b, where they generally occupy the majority of the cellular volume. Rod-shaped cells (R) were observed in the TEM images, where the vacuoles either appeared distributed throughout the volume or clustered around the center. Cross walls (CW) are present within the hyphae, separating the rod-like segments. Figure 7b shows a cross wall separating a living cell from a dying portion. The disintegrating hyphae cells (DC) show intracytoplasmic membranes in the form of isolated vesicles (V) in a granular cytoplasm and the separation of the plasma membrane from the cell wall. Further disintegration of cells was evident later in the process with disruption of the outer cell wall and emptying of cytoplasm and scattering of intracellular membrane vesicles throughout the degraded cells.

Acclimatization to production medium

At the time of inoculation of the nutrient deprived-amino acid rich production medium, the optical density of *S. venezuelae* at 600 nm (OD₆₀₀) was 0.805. It reached 0.823 after 3 hours of acclimatization



(Figure 8). The corresponding CFU concentration in production medium was 0.9×10^8 cell/mL and the TF yield was $0.225 \mu\text{mol}$. Therefore, the specific TF yield after 3 hours in the production medium was $0.25 \times 10^{-8} \mu\text{mol}/\text{CFU}$.

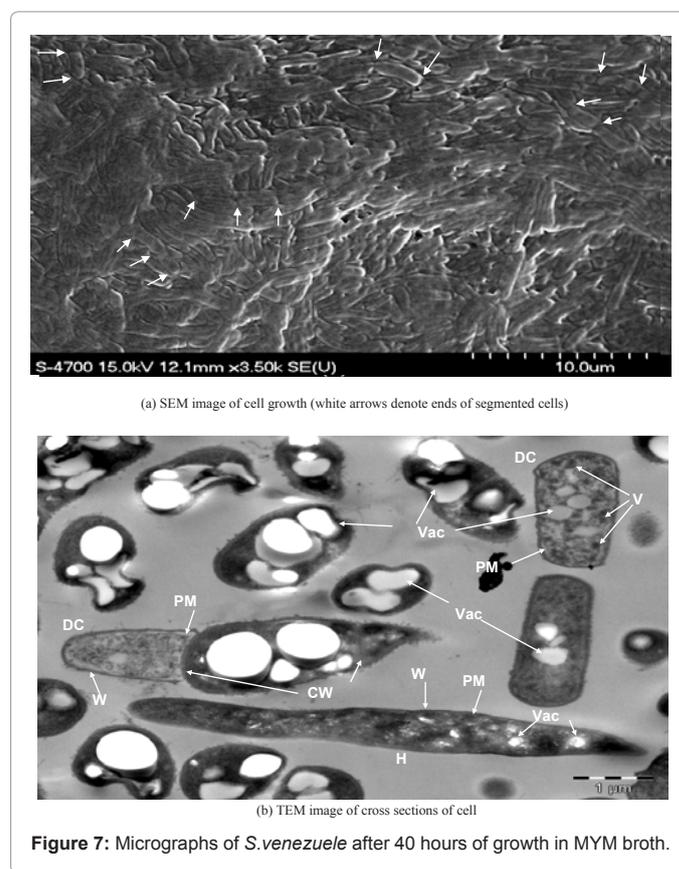
The SEM image of cells obtained 3 hours after inoculation of the production media (Figure 9a) showed a bed of filamentous hyphae. The cells did not appear uniform in width (width varied between 0.3 and 0.7 μm). Cell branching and septation were observed. Septation occurred along hyphae such that segmented chains of cells were observed with each segment having dimensions of approximately 0.4 by 0.7 μm (shown with white arrows in Figure 9a). TEM images of *S. venezuelae* cells obtained 3 hours after inoculation of the production media show long hyphae (H) with branching (B), disintegrated cells (DC) and rod-shaped cells (R) (Figure 9b,c). Lateral circular cross-

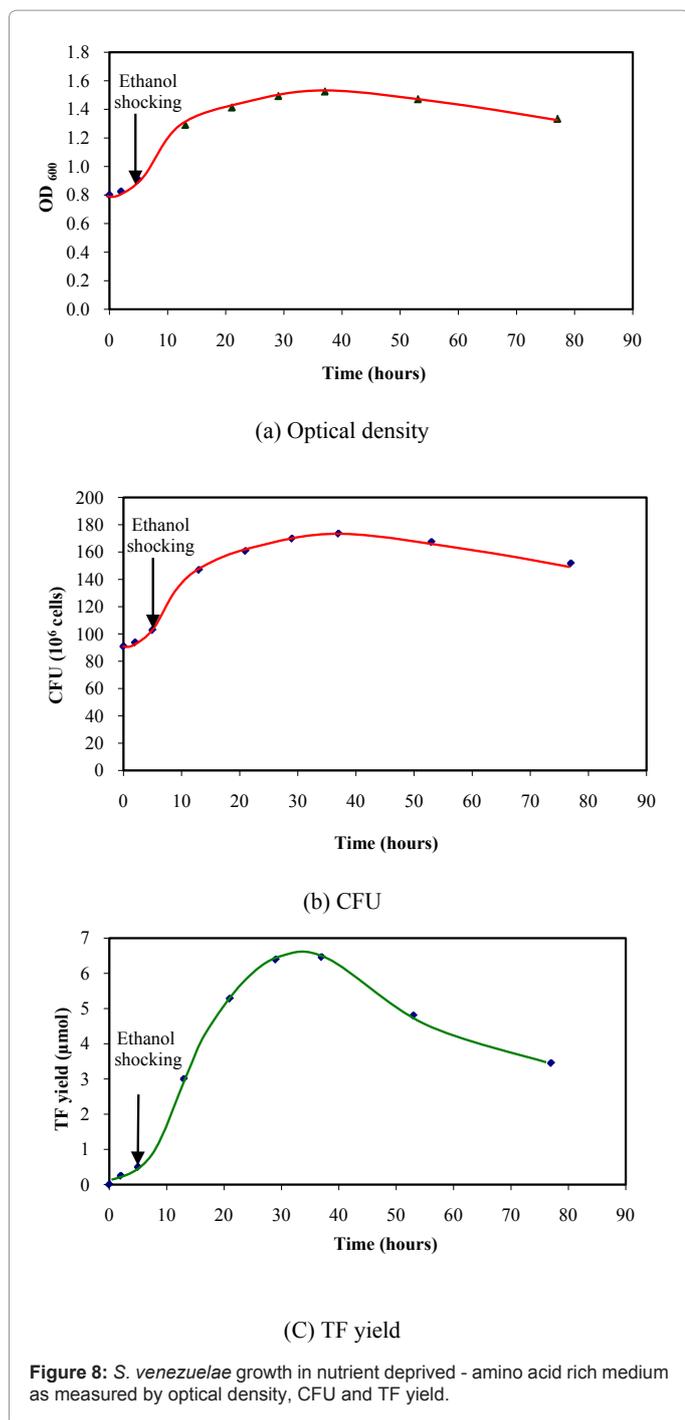
sections of thin hyphae were observed with diameters around 0.3-0.5 μm . There were virtually no electron-transparent vacuoles present in the thinner (width around 0.6 μm) hyphae (Figure 9b). In the rod-shaped cells (width around 0.8 μm), electron-transparent vacuoles were present and located near the center of the cells (Figure 9c). The vacuoles appeared less than 500 nm wide and occupied less than half of the cellular volume. Cross walls separating cells were present and rounding of the edges of the delimited cells (CW-R) was also observed.

Ethanol shock in production medium

The bacteria were shocked with ethanol after the 3 hour acclimatization period in production medium. Immediately after the ethanol shock, the optical density of *S. venezuelae* at 600 nm (OD_{600}) remained at 0.823 with corresponding CFU of 0.9×10^8 CFU/mL and TF yield of 0.25 μmol . Therefore, the specific TF yield was $0.28 \times 10^{-8} \mu\text{mol}/\text{CFU}$.

The TEM images (Figure 10a,b,c) show a similar population of cells as those observed during the acclimatization phase in the production medium. Long hyphae (H), disintegrated cells (DC) and rod-shaped cells (R) were observed. The long hyphae (Figure 10a) shows possible sites for branching (B) and an area that appears pinched (P). The pinched area is characterized by an absence of cytoplasmic material at its narrowest part so that only the outer wall of the hyphae separate two portions of cytoplasm-rich hyphae, where each portion is greater than 2 μm in length. Pinched areas also appear more frequently (Figure 10b), separating cytoplasmic-rich areas into areas around 1 μm in length, giving each segment an ovoid appearance. New tip growth (T) is present (Figures 10b,c), where the new tips appear much thinner (around 17-30 nm) than the cells they are emerging from. Electron-





transparent vacuoles are sometimes present in the cells (with the sizes below 500 nm), with many cells having no vacuoles present.

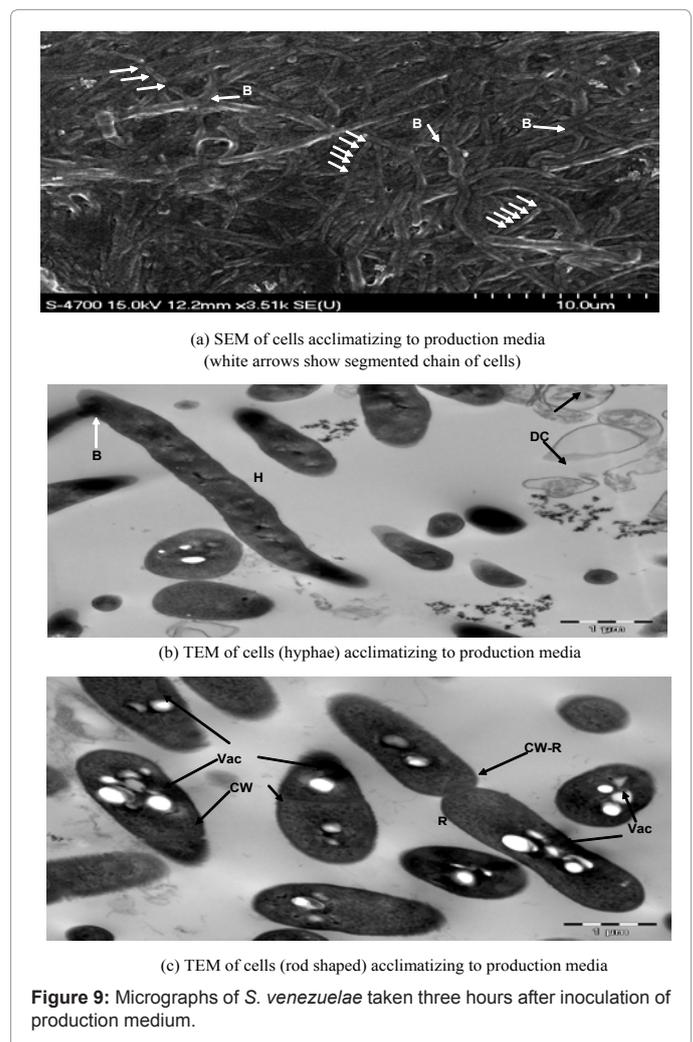
Production of jadomycin B

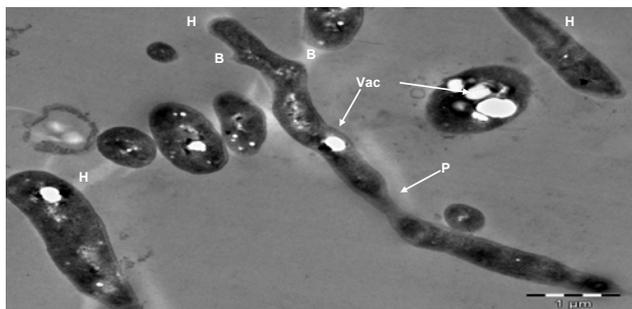
During jadomycin B production, *S. venezuelae* continued to grow in the production medium reaching a final optical density reading at 600 nm (OD_{600}) of 1.335 after a period of 75 hours since ethanol shock (78 hours of production). The highest OD_{600} was achieved after 37 hours of production. The number of CFU increased from 0.9×10^8 at the time of inoculation to 1.7×10^8 after 40 hours and then declined to

1.6×10^8 by the end of the production period (after 78 hours). The TF yield also increased from the initial value of $0.225 \mu\text{mol}$ to a maximum value of $6.5 \mu\text{mol}$ at the 37 hour and then declined to $3.5 \mu\text{mol}$ at the end of 78 hour production period. The specific TF yield increased from the initial value of $0.25 \times 10^{-8} \mu\text{mol}/\text{CFU}$ to a maximum of $3.82 \times 10^{-8} \mu\text{mol}/\text{CFU}$ at the 37 hour and then declined to $1.6 \times 10^{-8} \mu\text{mol}/\text{CFU}$ at the end of the 78 hour production period.

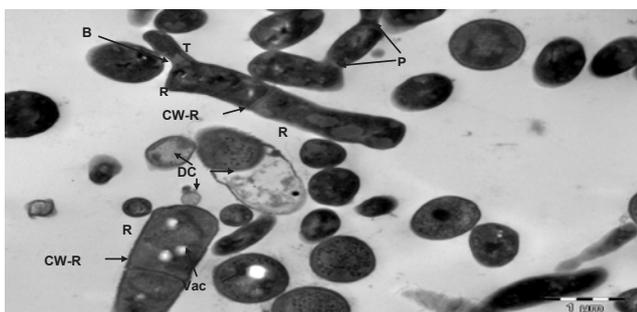
The SEM image of cells obtained 48 hours after ethanol shock in the production media (Figure 11a) showed a bed of filamentous hyphae, where most of the hyphae appeared segmented (as indicated by the white arrows highlighting segments on selected hyphae). The hyphae strands were typically segmented at intervals of $0.7 \mu\text{m}$. Cell branching was also evident (B). The cells did not appear uniform in width and the majority varied between approximately 0.3 and $0.5 \mu\text{m}$, although some slightly thicker cells were also present.

TEM images (Figure 11b,c,d) show long hyphae, disintegrated cells (DC) and evidence of spores (S). A germinating spore (GS) is shown, where a germ tube of $0.15 \mu\text{m}$ has emerged from the spore (Figure 11b). The long hyphae cells showed branching (B) at intervals of every 0.5 - $1.5 \mu\text{m}$ along the main branch. However, often the branches were not fully formed and appeared as short “bud”-like protrusions. There was evidence of pinching (P) along the hyphae, where the narrow pinched

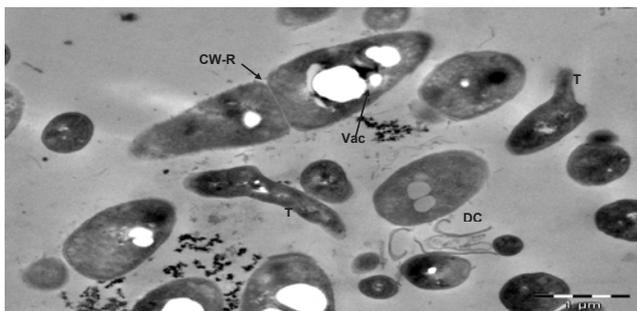




(a) TEM of cells (long hyphae) taken just after ethanol shock



(b) TEM image of cross sections of cells showing pinched areas



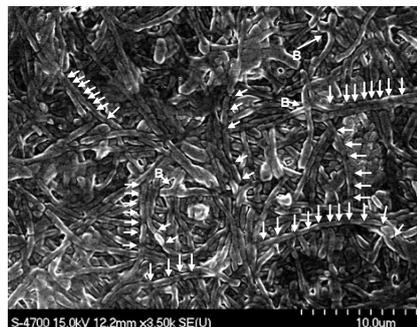
(c) TEM of cells taken just after ethanol shock showing tip growth

Figure 10: Micrographs of *S. venezuelae* just after ethanol shock in production medium.

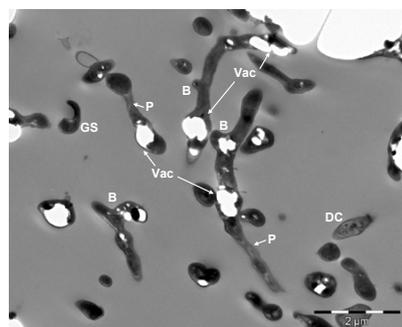
regions appeared devoid of cytoplasmic material. Figure 11c shows separation of the main hyphae cell from a spore-like cell (S), where the outer hyphal cell wall holds the spore in place. The pinched area appears flaccid, as the spore is not aligned with the cell and a bud-like protrusion is evident on the main hyphal branch where the spore has separated from the main branch. Further evidence of pinching is shown in Figure 11d where the segmented regions appear to be ovoid in shape. Electron-transparent vacuoles are seen in some cells, including the long hyphae, though it is not apparent how the vacuoles are distributed overall.

Jadomycin

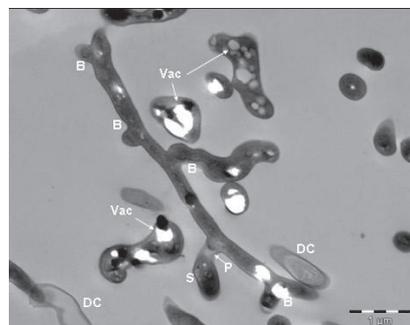
Jadomycin B production was monitored by measuring the absorbance at 526 nm (AU_{526}) as suggested by Jakeman et al. [6]. Jadomycin B was successfully produced as was indicated by the increase in AU_{526} and the change of the colour of production medium to dark orange. Jadomycin B production stopped after 37 hours from shocking (Figure 12). There was no further increase in the AU_{526} after 40 hours of production (37 hours after shocking with ethanol). The highest AU_{526}



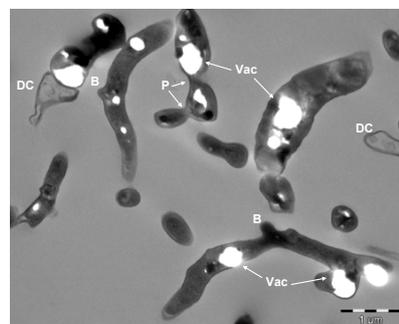
(a) SEM image of cells showing a bed of segmented hyphae



(b) TEM image of cross sections of cells showing evidence of branching vacuoles, disintegrating cells and germinating spores

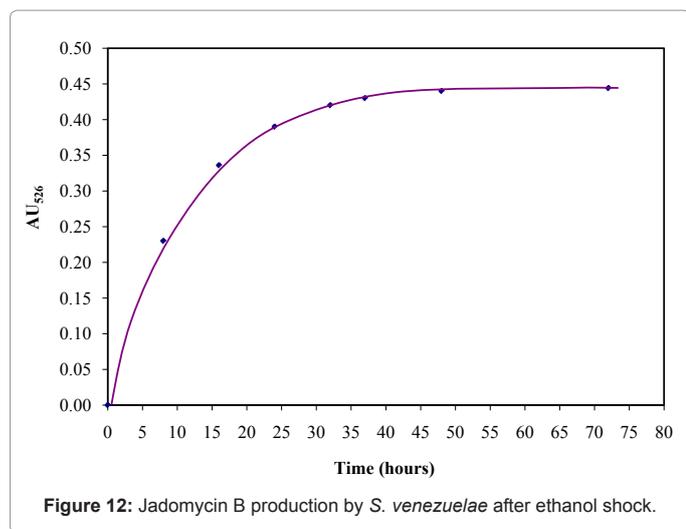


(c) TEM image of cross sections of cells showing separation of hyphae from spore cell



(d) TEM image of cross sections of cell showing segmented regions ovoid in like shape

Figure 11: Micrographs of *S. venezuelae* taken during jadomycin B production.



was 0.418. These results are in agreement with the highest OD₆₀₀ CFU/mL and TF yield which were obtained after 37 hour from shock with ethanol (Figure 8).

SEM images of purified jadomycin B powder (Figure 13) showed that the particles vary in size and shape. Figure 13a shows a small apparently circular piece of powder (approximately 3 μm in diameter) while Figure 13b shows a larger cluster of powder (approximately 40 μm by 40 μm in size), containing several particles of different shapes. The elemental analyses of the theoretical and purified jadomycin are shown in Table 3. The mean weight percentage of carbon, hydrogen, nitrogen and oxygen were 64.50±1.92, 5.10±0.76, 2.85±0.56 and 22.50±1.26, respectively. The obtained jadomycin powder appeared to have 97.95% purity.

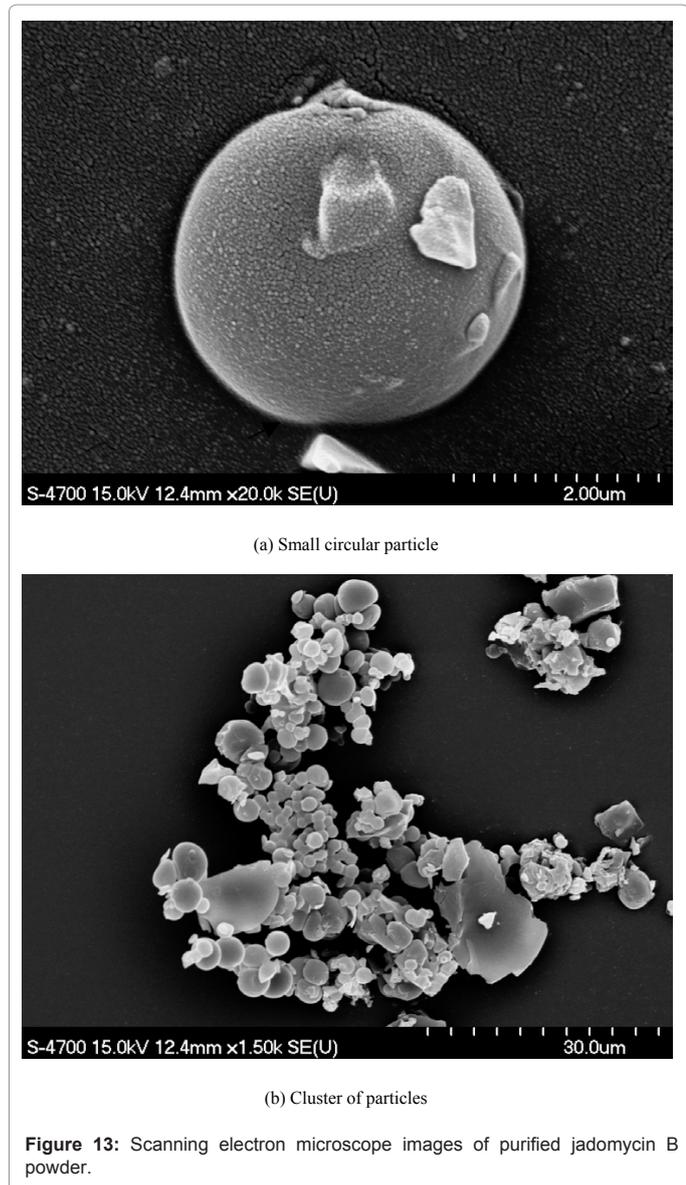
Discussion

S. venezuelae were exposed to four different environments during the process of jadomycin production. Significant changes in the activity, structure and morphology were observed during these stages as summarized in Table 4. These changes can be related to depletion of nutrients, shocking, and transition from vegetative cells to spore and the production of secondary metabolites. Figure 14 shows the level of cell activity as measured by the specific TF yield during growth and jadomycin B production periods.

During growth in MYM broth

S. venezuelae had the highest growth (2.25 x 10⁸ CFU/mL) during the growth phase in MYM broth. This was anticipated as the cells were provided with an ample supply of all nutrients required for sustained growth. The specific TF yield remained constant at 2.44 x 10⁻⁸ μmol/CFU during the growth period in MYM broth.

The SEM image of *S. venezuelae* taken during growth in the nutrient-rich MYM broth showed a dense bed of filamentous hyphae. The observed cell diameter of 0.5-1.0 μm is characteristic of the branching filaments from *Streptomyces* bacteria [13]. Bradley and Ritzl [14], reported that cross-walls are relatively rare in *S. venezuelae* vegetative hyphae. However, they also noted that the first step in sporogenesis is the simultaneous formation of many septa that allow the hyphae to divide into compartments, with each compartment eventually forming a spore. The segmentation via the cross-walls observed in the SEM (Figure 7a) would result in rod-like fragments of 2 μm in length, which would agree with the dimensions of the spores shown by Bradley and Ritzl [14], where the length varied between 0.5 and 1.8 μm with a width of 0.5-0.7 μm. Fragmentation of the hyphae would explain the rod-like cells that are also present in Figures 7b, 9c and 10b representing the stages of acclimatization to production media, ethanol shock and jadomycin production. While most actinomycetes do not sporulate in submerged culture [15] and it is considered an infrequent event [16], submerged sporulation has been observed in a number of cultures, including *S. griseus* [17-19], *S. viridochromogenes* [20], *S. roseosporus* [21], *S. coelicolor* [19,20], and *S. venezuelae* [16]. During the formation



Element	Experimental Weight (%)	Theoretical Weight (%)
Carbon	64.50±1.92	65.60
Hydrogen	5.10±0.76	5.64
Nitrogen	2.85±0.56	2.55
Oxygen	25.50±1.26	26.21
Others	2.05	
Total	100.00	100.00

Table 3: Elemental analysis of jadomycin B powder.

Parameter	Growth in MYM broth ^a	Acclimatization in production medium ^b	After ethanol shock	During jadomycin production ^c
OD ₆₀₀	1.750	0.823	0.823	1.444
CFU/mL	2.25 x10 ⁸	0.9x10 ⁸	0.9 x10 ⁸	2.67 x10 ⁷
TF (μmol)	5.5	0.225	0.250	0.135
TF (μmol/CFU)	2.44x10 ⁻⁸	0.25 x10 ⁻⁸	0.28 x10 ⁻⁸	3.68 x10 ⁻⁸
Hyphae width (μm)	0.5-1	0.3-0.7	0.3-0.7	0.3-0.5
Cross walls	√	√	√	x
Branching	x	√	√	√
Budding	x	x	√	√
Pinching	x	x	√	√
Vesicles	Large, numerous	Fewer	Fewer	Fewer
Spore septation	?	√	√	√
Germinating spores	x	x	?	√

^aAfter 40 hours of growth in growth media

^bAfter 3 hours of acclimatization in production medium

^cAfter 48 hours of jadomycin production

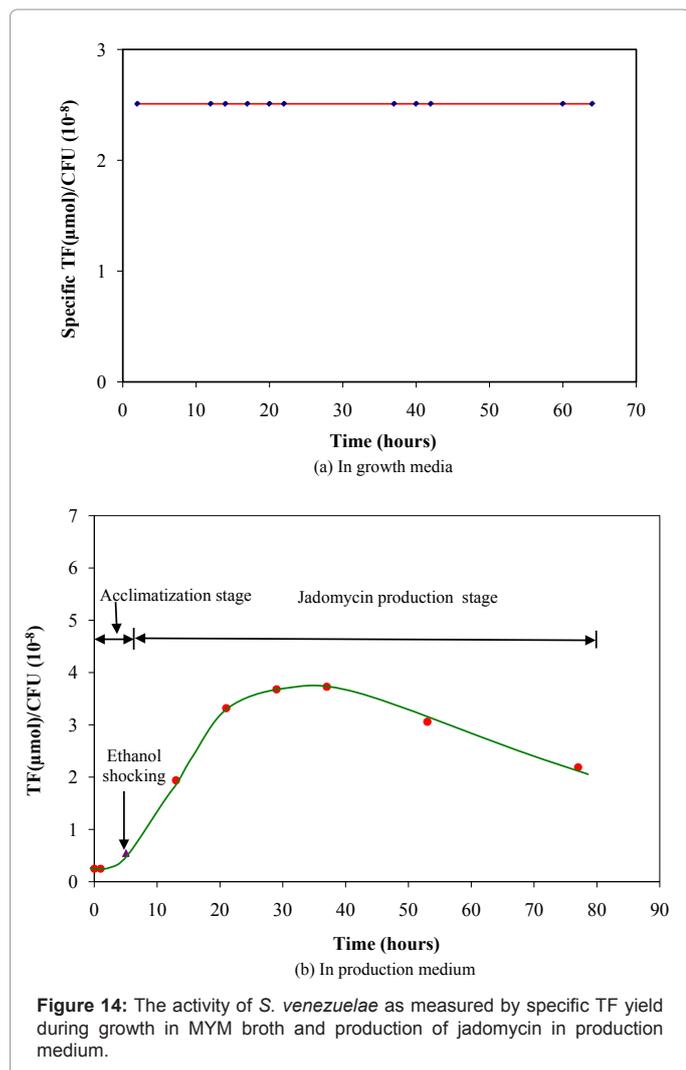
Table 4: Changes in activity, morphology and structure of *S. venezuelae* during the various stages of growth and jadomycin production.

of spores in submerged culture, it has been observed that apical sections of vegetative hyphae swell developing into long, moderately swollen sporogenic hyphae, which septate and develop into chains of immature spores that mature and then separate [22].

The TEM images of individual rod-like cells indicate that the longer sides of the rods were covered in a layer of amorphous material, whereas the shorter ends appeared smooth. This would support the idea that the rod-like cells were fragmented from hyphae, as the remnants of the outer component of the cell wall from the sporogenous hyphae frequently remain attached to the spore surface [14] which would correspond to the longer sides of the cell, while the shorter smooth sides would result from the newly-formed cross-walls which delimit the cells [23]. The physiological condition most often associated with spore formation is a nutritional downshift and it has been demonstrated that *S. venezuelae* requires nitrogen-limited conditions to sporulate [16]. At the time that the sample was taken (40 hours after inoculation of growth media), the cells were entering the stationary growth phase (Figure 6) and it is likely that cells experienced nutrient depletion that triggered spore formation.

The presence of disintegrating cells was evident in the TEM images with similar characteristics to that observed by Glauert and Hopwood [23]. Bradley and Ritzl [14], reported that in the stationary and death phases of growth, hyphal ghosts containing membranous vesicles were commonly observed during submerged cultivation. Rueda et al. [22] noted that sporogenic hyphae of *S. brasiliensis* growing in liquid medium consisted of two large compartments separated by a vegetative cross-wall, where the apical compartment would undergo multiple septation and subsequent development into a chain of spores, while the basal, non-sporulating compartment on the other side of the septum would degenerate and die.

The electron-transparent vacuoles present in the TEM image (Figure 7b) are similar to those observed for *S. lividans* by Packter and Olukoshi [24] during the late stationary growth phase, where the discrete structures were of various sizes, had no internal structure and occupied up to approximately 80% of the total cell volume at that time. They attributed the vacuoles as comprising of neutral lipids containing saturated fatty acids and while glycogen, polyhydroxybutyrate (PHB) and polyphosphates provided typical energy reserve compounds in bacteria, they reported that the accumulation of triacylglycerol (TAG) during the late stages of submerged growth was a unique characteristic of *Streptomyces* spp. Ranade and Vining [25], reported that small amounts of PHB were accumulated by *S. venezuelae* during exponential growth in media supporting chloramphenicol production, though the compound disappeared from the mycelium during the stationary growth phase. Glycogen and lipids accumulated in the mycelium during



the stationary phase only after nitrogen was depleted, providing the principal storage compounds in *S. venezuelae*. The longitudinal cross-section of a hyphae cell in Figure 7b appears to have very little storage material present, indicating that it is undergoing rapid growth, while the other structures which are likely cross-sections of swollen hyphae indicate that these are older hyphae with storage of TAG. Ranade and Vining [25], also noted that both glycogen and PHB were absent from spores. It is unclear whether the rod-like cells are spores or vegetative hyphae fragments. This is because multi-septation characteristic of spore formation has been observed, however the rod-like cells appear to have storage material present as vacuoles can be seen. The interpretation of Glauert and Hopwood [23], of a *Streptomyces* spore being a segment of hypha surrounded by a thickened hyphal wall may be useful in this case. Bradley and Ritzl [14], noted that the cell wall of a mature spore of *S. venezuelae* is around 30 nm, whereas the cell wall in vegetative hyphae is typically around 15 nm. It is possible that the rod-like cells may be a form of immature spore.

During acclimatization to production medium

Before the ethanol shock in the production medium, the population size was 0.9×10^8 CFU/mL. The specific TF yield was relatively low (0.25×10^{-8} $\mu\text{mol}/\text{CFU}$) compared to that of vegetative cells grown in MYM broth.

Hyphae viewed under the SEM ranged in diameter from 0.3 to 0.7 μm which was thinner on average than that observed in the growth media. There was extensive branching and crossing of hyphae, which was not as evident in the growth media. In addition, the septation characteristic of spore formation was observed along the length of the hyphae. However, the cross-walls appeared at shorter intervals resulting in segments of 0.7 μm in length rather than 2.5 μm . The septation and thinning of mycelia are characteristics of ageing *Streptomyces* [26]. The TEM images provide insight into the changes occurring within the cells during the acclimatization period. There were no vacuoles present in the long hyphae (Figure 9b), indicating that the hyphae were in a rapid growth phase, which coincides with Figure 8b where the cells are just entering the exponential growth phase. The rod-shaped cells in Figure 9c show that the energy reserve vacuoles occupy a smaller volume within the cells and appear less numerous, which may signify a depletion of TAG, as the cells use their energy reserve to become more active metabolically in adjusting to the new environment. Figure 8b indicates that there is an increase in CFUs at this stage. As *Streptomyces* form CFUs from viable fragments of mycelium or outgrowths from spores [13], which indicates that the cells are actively growing. The remains of cells that have disintegrated were visible at this stage, thus the rate of cellular growth was greater than the rate of cell death.

After ethanol shock

Immediately after the administration of the ethanol shock, the *S. venezuelae* population size did not change in number and remained at 0.9×10^8 CFU/mL but the specific TF yield slightly increased from 0.25×10^{-8} $\mu\text{mol}/\text{CFU}$ to 0.28×10^{-8} $\mu\text{mol}/\text{CFU}$. Cells viewed under both the SEM and TEM appeared largely similar to the cells viewed just prior to the ethanol shock. However, there were some additional features observed. The TEM images showed pinching of vegetative hyphae, which appeared to segment the hyphae as portions of the hyphae became devoid of cytoplasmic material, resulting in "empty" flaccid regions. According to Pons et al. [26], *Streptomyces* tend to lose cellular content with ageing, which results in empty compartments. Segmentation by pinching seemed different from segmentation by the formation of cross-walls, as the cross-walls were typically formed

between areas rich in cytoplasmic material, where the hyphae appeared turgid. In Figure 10b, the pinching appears to form a chain of spore-sized cells. Bradley and Ritzl [14], reported that on solid media *S. venezuelae* developed sporulating aerial hyphae comprised of mature spores that were held in chains by a thin outer sheath. In their study of *S. brasiliensis*, Rueda et al. (2001) observed that sporogenic hyphae cultivated in submerged liquid media were morphologically similar to aerial hyphae and developed into chains of spores in a comparable manner.

During jadomycin production

S. venezuelae population increased following the ethanol shock reaching to a maximum of 1.745×10^8 $\mu\text{mol}/\text{CFU}$ after 32 hours, after which there was a significant decrease in population size. The TF yield followed a similar trend but the decrease in TF yield was much faster than the decrease in population size. The specific TF yield (a measure of cell activity) increased following ethanol shock and reached a maximum of 3.68×10^{-8} $\mu\text{mol}/\text{CFU}$ after 32 hours following shocking and then declined reaching 2.35×10^{-8} $\mu\text{mol}/\text{CFU}$ by the end of the experiment.

S. venezuelae produces two antibiotics choramphenicol and jadomycin, neither of which is produced in rich media [27]. Choramphenicol production is associated with nitrogen metabolism and high rates of production are observed when poorly assimilated nitrogen sources are available, which also results in a slower microbial growth rate [28]. Jadomycin is only produced when an additional stress (e.g. heat or ethanol) is applied to nutritionally unbalanced cultures [4]. One of the genes involved in jadomycin biosynthesis has also been found to affect morphological differentiation [27]. In the study by Wang and Vining [27], disruption of the gene resulted in a reduced growth rate of *S. venezuelae* during submerged cultivation and an altered growth morphology in minimal media. Also, sporulation of the mutants with the disrupted gene appeared less abundant and slower than the wild-type. In the present study, morphological differentiation of the bacteria was observed during jadomycin production. Spores are associated with this phase and it is possible that the production of jadomycin occurs as the spores are formed from the vegetative mycelia, for example during the septation process. It is interesting to observe that after the ethanol shock and during jadomycin production, septation by pinching appears more common where the septated filaments appear less turgid and the rigid-looking cross-walls that were present in cells grown in the nutrient-rich growth media are not observed.

In this present study, cells were still dividing in the production medium for up to 32 hours after ethanol shock, as the CFUs increased. This indicates that viable fragments of mycelium and/or germinating spores were present [13]. It was noted that samples taken from the production media at 32 hours after ethanol shock required greater than 48 hours of incubation for CFU development on MYM agar, whereas only 24 hours of incubation were required for samples plated from those grown in MYM broth, acclimatized to production media prior to ethanol shock and immediately after ethanol shock. It is unknown whether this delay in visible colony growth on MYM agar was the result of CFU formation from spores or from extremely stressed vegetative cells. The SEM and TEM images indicated that the vegetative cells undergoing septation and germinating spores are present within the liquid media. Therefore, it is likely that ungerminated spores are present as well. In any case, it is evident that the cells present in the sample of production media 32 hours after ethanol shock are morphologically different than the cell population present in the other samples, so it is not surprising that the time for colony formation on agar would differ.

The optimization of the ethanol shock to maximize jadomycin is important when considering scale-up. If this process could be improved, higher jadomycin yield may be achieved in a more timely fashion. In larger volume reactors, the stress may not be uniformly applied to all cells. In this case, possible ways of improving jadomycin yield may be to (a) administer a larger ethanol shock and/or (b) administer a second shock during the production phase. However, in order to assess the efficiency of the stress application methods, it is necessary to determine when the jadomycin is produced and whether jadomycin production is associated with the stressed vegetative hyphae, the process of septation or the germination of spores. Once this is determined, strategies to increase the efficiency and improve methods for applying the environmental stress can be studied.

Jadomycin

From the results obtained in this study, it was not possible to detect jadomycin B within cells or cell vesicles during the production phase. It is probable that jadomycin is produced extracellularly by excreted enzymes, as opposed to intracellularly by the uptake of isoleucine and glucose, which are the media components required to produce jadomycin B molecules. It has been reported that species of the *Streptomyces* genus are able to metabolize many molecules, including amino acids extracellularly by hydrolytic enzymes [29]. The white pellet (white cells) observed from centrifugation of production medium during the jadomycin production phase further supports the idea that jadomycin B is produced externally to the cells, as jadomycin is a highly coloured compound. While it is possible an extracellular hydrolytic enzyme is responsible for the incorporation of isoleucine into the jadomycin B structure, Rix et al. [30] reported that no such enzyme had yet been identified. It is, therefore, possible that the cells were highly efficient at excreting the secondary metabolites. Further studies are needed to confirm either hypothesis.

The jadomycin B concentration reached a maximum and plateaued approximately 42 hours after the ethanol shock. During the production of secondary metabolites, cells are no longer using nutrients for growth but rather for the synthesis of defensive compounds. Several researchers have suggested a possible pathway for jadomycin B production involving the oxidation of the 5,6-bond on the angucycline intermediate, which then allows for the incorporation of isoleucine [1,30]. Dehydrogenase enzymes oxidize organic matter; therefore, it is not surprising that an increase in dehydrogenase activity was observed during the production of jadomycin B.

The chemical formula of jadomycin B is $C_{30}H_{31}NO_9$, with a molecular weight of 548 g/mol [3]. The percentages of carbon, hydrogen, nitrogen and oxygen according to the chemical formula are 65.67, 5.65, 2.55 and 26.23 wt.%, respectively. The weight percentages obtained in the study for carbon, hydrogen, nitrogen and oxygen were very close to those reported for jadomycin. The purity of jadomycin powder was 97.95%.

Conclusion

The dehydrogenase activity measurement assay was used to investigate changes in activity of *S. venezuelae* under different environmental conditions. The lowest specific TF yield (0.25×10^{-8} $\mu\text{mol}/\text{CFU}$) was noted during the acclimatization period in production medium. The specific TF yield ($\mu\text{mol}/\text{CFU}$) increased for the first 32 hours following the ethanol shock in production medium to a maximum yield of 3.68×10^{-8} , after which a gradual decline was observed to a final measurement of 2.35×10^{-8} at the end of the experiment. The trend of the specific TF yield ($\mu\text{mol}/\text{CFU}$) was directly related to jadomycin B production. Jadomycin B production reached a

maximum about 42 hours after the ethanol shock as the cells continued to produce small amount of jadomycin few hours after the TF yield reached its maximum.

Structural changes in *Streptomyces venezuelae* were observed under scanning and transmission electron microscopes. After the ethanol shock was applied, the mycelia appeared thinner and septation (characteristic of sporulation) was evident resulting in chains of segmented cells with a "pinched" rather than turgid appearance. Germinating spores were also observed. Notably smaller and fewer storage vacuoles existed during the acclimatization phase and after the ethanol shock phase, indicating that cells were utilizing the storage lipids accumulated at the end of the growth phase.

The application of environmental stress (ethanol shock) is a key step in inducing the cells to produce jadomycin. Therefore, developing methods to effectively apply the stress to the cell population would maximize jadomycin production. The metabolic activity of the cells is associated with their physiology and structure. As the ethanol shock did not cause 100% of the cells to change their morphology immediately, a more effective stressing mechanism is needed to maximize jadomycin production from the cell population. The dehydrogenase activity assay for measuring the activity of *Streptomyces venezuelae* is an important tool that can be used to optimize the timing of ethanol shock to initiate jadomycin B production and provide a quantitative measure for evaluating the success of the shock. The structural changes that were observed in the bacteria during the growth and jadomycin production phases give a qualitative measure that can be used to assess the effectiveness of the environmental stress. Thus, the dehydrogenase test and morphological observations will prove to be invaluable for designing a procedure to achieve optimal yields of the useful antibiotic.

The elemental analysis provided acceptable data for verifying jadomycin B production. However, the poor understanding of jadomycin B biosynthesis by *Streptomyces venezuelae* emphasizes the need for further investigation into the phenomenon responsible for forming the useful compound. Points of interest requiring further exploration are the exact mechanism of synthesis, the involvement of enzymes, and the location of the synthesis. Potential means of investigating the location of synthesis include using an isoleucine fluorescence tag and viewing cell slices under a transmission electron microscope or lysing cell membranes during the production phase to examine cell contents. A kinetic study on the uptake of glucose from the production medium may be more feasible and provide insight into how isoleucine is behaving during jadomycin B production, as both molecules are found in the chemical structure of jadomycin B. This may provide an idea as to how jadomycin B production can be optimized.

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