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A Dehydrogenase Activity Test for Monitoring the Growth of *Streptomyces Venezuelae* in a Nutrient Rich Medium

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Abstract

Jadomycin is a novel antibiotic that has shown activities against bacteria, yeasts and fungi as well as cytotoxic properties to cancer cells. Because of the wide range of its inhibitory actions, jadomycin shows promise as a novel antibiotic and cancer treatment drug. *Streptomyces venezuelae* are aerobic bacteria that are capable of producing jadomycin when shocked by alcohol in a nutrient deprived amino acid rich medium. The size of the bacterial population that is transferred from the growth medium to the production medium can significantly affect the jadomycin yield. Therefore, the number of transferred bacteria must be accurately measured. In this study, a dehydrogenase activity measurement test was developed for *S. venezuelae* using triphenyl tetrazolium chloride (TTC) to measure the cell growth and activity in maltose-yeast extract-malt extract (MYM) broth. The dehydrogenase activity was determined by measuring the visible color changes of the TTC to triphenyl formazan (TF). The test conditions which included extraction solvent, number of extractions, incubation time, incubation temperature and medium pH were evaluated. The results showed that the triphenyl formazan was related to the number of cells. Methanol was better able to permeate the cells and extract higher amount of TF than ethanol. The amount of TF increased with the number of extractions for both solvents. A lower medium pH and/or lower temperature produced the highest amount of TF. The best test conditions that produced the highest TF yield were three extractions using methanol after an incubation time of 1 hour at a temperature of 30ºC and a medium pH of 6.

Keywords: Dehydrogenase; Triphenyl tetrazolium chloride (TTC); Jadomycin; *Streptomyces venezuelae*

Introduction

Jadomycins are a novel group of antibiotics that are produced by *Streptomyces venezuelae*. They exhibit biological activity against bacteria and yeast and demonstrate cytotoxicity against cancer cells [1]. *S. venezuelae* are typically grown in a nutrient rich medium of maltose-yeast extract-malt extract (MYM) broth prior to their introduction to a nutrient-deprived, amino acid-rich production medium [2,3]. Once transferred to the production medium, an environmental shock (ethanol or heat) is applied to induce the bacteria to produce jadomycin [4]. The size of the bacterial population that is transferred from the growth medium to the production medium can significantly affect the jadomycin yield [5]. Therefore, in order to standardize and improve the reproducibility of the jadomycin production process, the number of live bacteria that are transferred and subsequently shocked must be accurately determined.

Bacterial counts can be determined with various techniques such as plate counting [6] turbidity measurement [7], microscope enumeration [8] and dehydrogenase activity measurement (Knight et al.). The dehydrogenase activity measurement technique has the advantage over other methods in being able to quantify the number of live cells that are present in the medium [9-11] and it can be employed in a relatively short time and at a low cost [12].

The dehydrogenase activity test is based on the principle that dehydrogenase enzymes are produced by all living cells and the extent to which this enzyme group oxidizes organic matter can be related to the number of live cells present [12]. This group of enzymes transports electrons and hydrogen through a chain of intermediate electron carriers to a final electron acceptor (oxygen), resulting in the formation of water [13-16]. The activity of the co-enzymes Nicotinamide Adenine Dinucleotide (NAD) and Flavin Adenine Dinucleotide (FAD) which act as intermediate electro acceptors can be measured by the visible color or change of a dye such as triphenyl tetrazolium chloride (TTC) [12].

The process of measuring dehydrogenase activity involves incubating the sample in the presence of triphenyl tetrazolium chloride and an electron-donating substrate[12]. In its oxidized form, TTC is colourless, but in the presence of dehydrogenase enzymes TTC is reduced to triphenyl formazan (TF), a red water insoluble compound [12, 17-19]. The TF is retained within microbial cells and can result in highly coloured colonies when grown on agar plates [18]. The mechanism of the process is summarized in Figure 1.

Triphenyl formazan can be extracted from cells using a solvent and the concentration is determined colorimetrically by measuring the optical density at 484 nm [12]. The use of a dehydrogenase activity measurement test using TTC to measure the quantity of living cells has great potential as a quick tool for determining the optimal time to shock a population of *S. venezuelae* and start the production of jadomycin. However, the amount of TF extracted depends on number of extractions, extraction solvent, incubation time, incubation temperature, and medium pH [20].The purpose of this study was to develop a dehydrogenase activity measurement test for *S. venezuelae* that could be used to quantify the live bacterial cells. The specific objectives were to: (a) investigate the applicability of the TTC-test for measuring dehydrogenase activity in *S. venezuelae* and (b) determine the ideal test

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conditions (extraction solvent, number of extractions, incubation time, incubation temperature and medium pH).

Experimental Materials

Reagents

Tris (hydroxymethyl-aminomethane) buffer was used to control the pH of the samples and triphenyl tetrazolium chloride (TTC) was the tetrazolium salt used for the dehydrogenase test. A TTC-glucose reagent (1 g glucose and 2g TTC dissolved in 100 mL distilled water) was prepared and stored in the dark at 4°C until used. Triphenyl formazan (TF) was used to establish a standard curve for absorbance (OD_{484}) vs TF concentration. Alcohols (ethanol and methanol) were used to extract TF from the cells. The Tris (hydroxymethyl-aminomethane), 2, 3, 5-triphenyl tetrazolium chloride (TTC) and 2, 3, 5-triphenyl formazan (TF) were obtained from Sigma-Aldrich (Oakville, Ontario, Canada) and the glucose was obtained from BioShop (Burlington, Ontario, Canada). Ethanol and methanol were obtained from Fisher Scientific (Montreal, Quebec, Canada).

Media preparation

Maltose-yeast extract-malt extract (MYM) agar and broth were used to cultivate *Streptomyces venezuelae*. The compositions of MYM agar and broth are shown in Table 1. All media components were obtained from BioShop (Burlington, Ontario, Canada). The media components were dissolved in distilled water then autoclaved (Sterile ax, Thermo Fisher Scientific, Ottawa, Ontario, Canada) on the liquid setting (121°C and 20 Pa) for 15 minutes. The autoclaved agar was stored at 65°C to prevent solidification.

Bacteria

An initial starter plate of *Streptomyces venezuelae* ISP5230 was obtained from the Jakeman Laboratory, College of Pharmacy, Dalhousie University (Halifax, Nova Scotia, Canada) and stored at 4°C. The surface growth was used to inoculate maltose-yeast extract-malt extract (MYM) agar plates or flasks with MYM broth as needed.

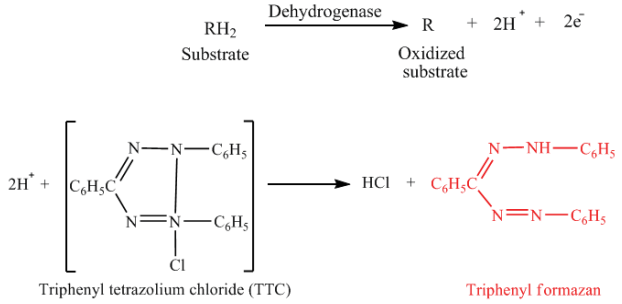


Figure 1: Mechanism showing the role of dehydrogenase in the reduction of triphenyl tetrazolium chloride (TTC) to triphenyl formazan (TF).

Component	Quantity (g/L distilled water)	
	Agar	Broth
Maltose	4.0	4.0
Yeast Extract	4.0	4.0
Malt Extract	10.0	10.0
MOPS	1.9	1.9
Agar	15.0	-

Table 1: MYM media components.

Experimental Procedure

Triphenyl formazan (tf) standard curve

A standard curve was developed to determine the concentration of TF corresponding to an absorbance measurement at 484 nm. A stock solution of 0.2 µmol/mL was prepared by dissolving 0.03 g TF in 500 mL methanol. The stock solution was diluted with methanol to produce 11 solutions with TF concentrations from 0.004 µmol/mL to 0.1 µmol/mL. The absorbance of each solution was measured using a spectrophotometer (Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada) at a wavelength of 484 nm. The absorbance readings were plotted against the TF concentration of the prepared solutions as shown in Figure 2. The following linear best-fit equation ($R^2 = 0.98$) was determined:

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

where:

AU_{484} is the absorbance reading at 484 nm

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

Microbial growth

Three 250 mL shake flasks were each filled with 175 mL of MYM broth, plugged with foam caps, covered with aluminum foil and autoclaved (SterileMax, Thermo Fisher Scientific, Ottawa, Ontario, Canada) at 121°C and 20 Pa for 15 minutes. The flasks were then inoculated with *S. venezuelae* and incubated in a controlled environment shaker (25 Incubator Shaker, New Brunswick Scientific, Edison, New Jersey, USA) at 30°C and 250 rpm. Each flask was sampled at 0, 2, 12, 14, 21, 23, 38, 40, 42, 60 and 64 hours after inoculation and the extent of cell growth was monitored over the period of 64 hour by measuring the optical density at 600 nm (OD_{600}), the number of colony forming units (CFU) and the triphenyal formazan yield (TF).

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

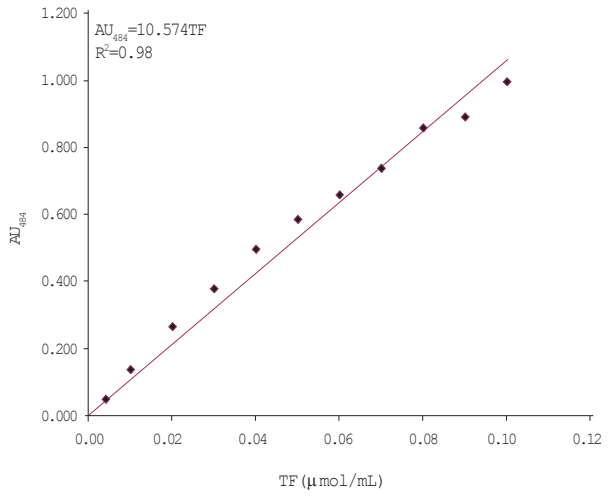


Figure 2: TF standard curve.

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 7 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 ethanol 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).

Optimization experiments

Experiments were conducted to determine the optimum test conditions that would reduce the most TTC to TF and extract the highest amount of TF from *S. venezuelae* during growth in MYM media. The dehydrogenase activity test parameters included: solvent type, number of solvent extractions, incubation time, incubation temperature and medium pH. The values for each parameter are shown in Table 2. The study was carried out in two phases as shown in Figure 3. First, initial experiments were conducted to determine the optimal number of solvent extractions and best solvent type. Then, the best solvents and optimum number of extraction obtained from the initial experiments were used in further experiments to determine the best time, temperature and pH.

Number of extractions

To evaluate the effect of the number of extractions on the TF yield from each flask, samples were taken from the growing culture at 21, 37, 47, 62 and 64 hours after inoculation. At each sampling time, 1 mL aliquots were transferred into four test tubes (tests were carried out in triplicates with a control for each sample). Tris buffer (2.5 mL) was added to all test tubes. Then, 1 mL of TTC/glucose solution was added to each of the sample test tubes (1 ml of distilled water to control test tubes). The pH was adjusted to 6 using 1 N HCl. The test tubes were gently swirled to mix the contents, incubated in a controlled temperature oven at 50°C (Isotemp Oven, model 630 F, Fisher Scientific, Ottawa, Ontario, Canada) for 1 hour. Samples were then centrifuged (IEC CentraCL2, Thermo Electron Corporation, Mississauga, Ontario) for 10 minutes to separate the cells from the liquid media. The supernatant was discarded and 2.5 mL of ethanol was added to the cells. All samples were vortexed (Thermolyne Maxi Mix, Thermolyne Corporation, Hampton, New Hampshire, USA) to aid in the extraction of TF (red colour) from the cells. Samples were centrifuged again, the supernatant decanted and absorbance measured at 484 nm using the control to zero the spectrophotometer (Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada). A second extraction with ethanol was carried out, the supernatant was combined with that from the first extraction and the absorbance was measured. A third extraction with ethanol was carried out, the supernatant combined with those from the previous two extractions and the absorbance was measured.

Extraction Solvent	Number of Extractions	Incubation Time (hour)	Incubation Temperature (°C)	Medium pH
Methanol	1	1	22	6
Ethanol	2	2	30	7.5
	3	3	40	9
		4	50	

Table 2: Dehydrogenase activity assay conditions.

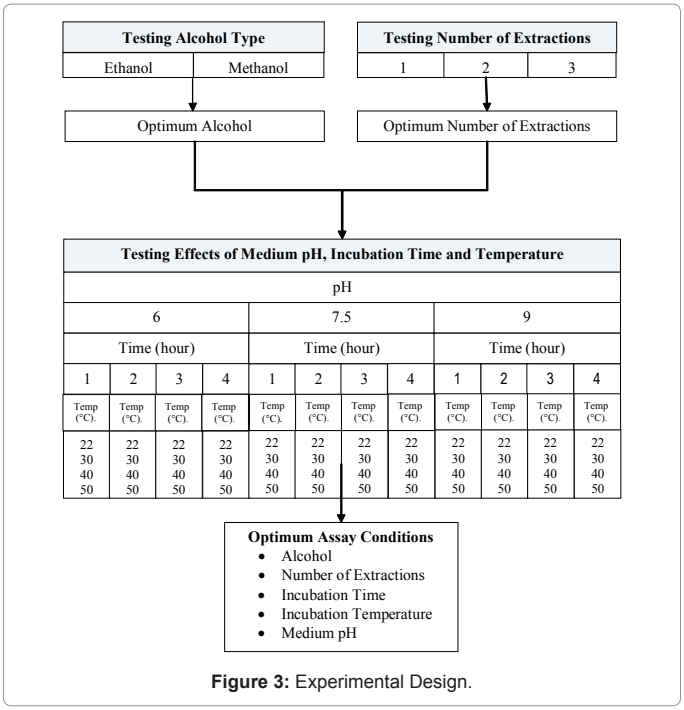


Figure 3: Experimental Design.

Type of extraction solvent

To evaluate the effectiveness of solvent in extracting TF, samples were taken from the growing culture at 21, 37, 47, 62 and 64 hours. At each sampling time, aliquots (1 mL) were transferred from each flask into the two groups of test tubes (ethanol and methanol). Tests were carried out in triplicates and a control for each flask sampled and solvent tested. Tris buffer (2.5 mL) was added to all the test tubes and 1 mL of the TTC/glucose solution was added to the sample test tubes (or 1 mL of distilled water to control test tubes). The pH was adjusted to 6 using 1 N HCl. The test tubes were gently swirled to mix the contents and incubated at 50°C for 1 hour in a temperature controlled oven (Isotemp Oven, model 630F, Fisher Scientific, Ottawa, Ontario, Canada). Samples were then centrifuged (IEC CentraCL2, Thermo Electron Cor-

poration, Mississauga, Ontario) for 10 minutes and the supernatant discarded. Extraction of TF was carried out three times using 2.5 mL of either methanol or ethanol. After each addition of solvent to the cells, the samples were vortexed, centrifuged and the supernatant decanted. The absorbance of the supernatants collected from three extractions for each solvent for each solvent was measured at 484 nm using a spectrophotometer (Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada).

Incubation time, temperature and medium ph

After 64 hours of growth, the contents of all flasks were combined into a 1 L flask and refrigerated at 4°C until required. For each incubation temperature investigated (22°C, 30°C, 40°C and 50°C), three medium pH values (6, 7.5 and 9) and four incubation times (1, 2, 3 and 4 hours) were tested. The resulting 48 tests were carried out in triplicate with a control. For all tests, 1 mL aliquots of the MYM broth with *S. venezuelae* growth were added to test tubes. The pH was adjusted to 6, 7.5 or 9 using 1 N HCl or NaOH as needed. Tris buffer (2.5 mL) and TTC/glucose solution (1 mL) were added to each tube. Tubes were manually swirled to mix contents and incubated for either 1, 2, 3, or 4 hours. Tubes were incubated at 22°C or 30°C in controlled environment incubator (Model 2020, VWR International, Cornelius, Oregon, USA), and at 40°C or 50°C in temperature controlled oven (Isotemp Oven, model 630F, Fisher Scientific, Ottawa, Ontario, Canada). Samples were then centrifuged (IEC CentraCL2, Thermo Electron Corporation, Mississauga, Ontario) for 10 minutes and the supernatant discarded. Extraction of TF was carried out three times using 2.5 mL methanol each time. After each addition of solvent to the cells, the samples were vortexed, centrifuged and the supernatant decanted. The supernatants obtained from the three extractions were combined after each extraction and the absorbance was measured at 484 nm using a spectrophotometer (Genesys 20, Thermo Scientific, Mississauga, Ontario, Canada).

Results

Microbial growth

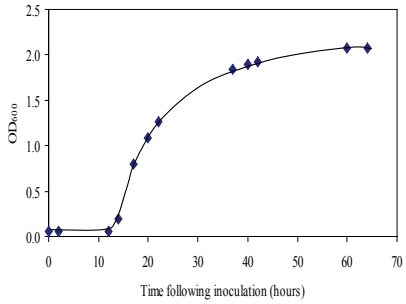
The microbial growth as determined by measuring optical density at 600 nm (OD_{600}), the number of colony forming units (CFU) and the triphenyl fromazan (TF) yield. The results are presented in Figure 4. There was an initial lag period followed by exponential growth phase. The lag period and specific growth phase were determined graphically according to the procedure described by [15] as shown in Figure 5. The lag period and specific growth were 10.3 hour and 0.3 h^{-1}, respectively.

Number of extractions

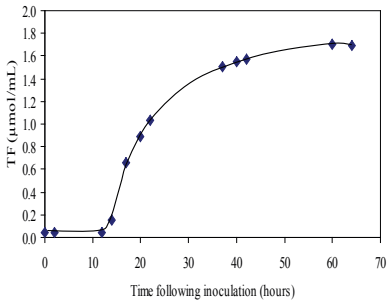
In order to test the effect of the number of extractions on the final TF yield from *S. venezualae* cells grown in MYM broth, one, two and three extractions were carried out at a medium pH of 6, an incubation time of 1 hour and an incubation temperature of 50°C. The results presented in Figure 6 showed higher TF yields with increasing number of extractions at all sampling times (different population sizes). The results also showed that the stationary growth phase was reached after the 60 hours of growth.

Type of extraction solvent

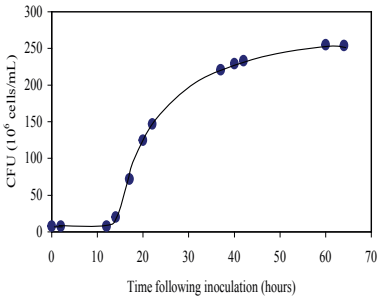
Ethanol and methanol were used to extract TF from samples taken during the growth of *S. venezuelae* cells. The test was carried out using three extractions at a medium pH of 6, an incubation temperature of 50°C and an incubation time of 1 hour. The TF results shown in Figure 7 indicated that both solvents showed an increase in TF yield over the time as the number of bacteria increased. The results also showed that the TF extracted by ethanol and methanol started to decline after 60 hours of growth indicating the start of the stationary growth phase.



(a) Optical density



(b) TF yield



(c) CFU

Figure 4: *S. venezuelae* growth measured by optical density, TF yield and CFU.

Medium pH

Figures 8 and 9 show the effect of pH on TF yield at varying incubation temperatures and incubation times, respectively. All plots display a similar concave shape with the pH value of 7.5 resulting in the lowest TF yield and the pH of 6 resulting in the greatest TF yield at all incubation times and temperatures. However, longer incubation time and lower temperature resulted in slightly higher TF yields.

Incubation time

Figures 10 and 11 show the effect of incubation time on TF yield

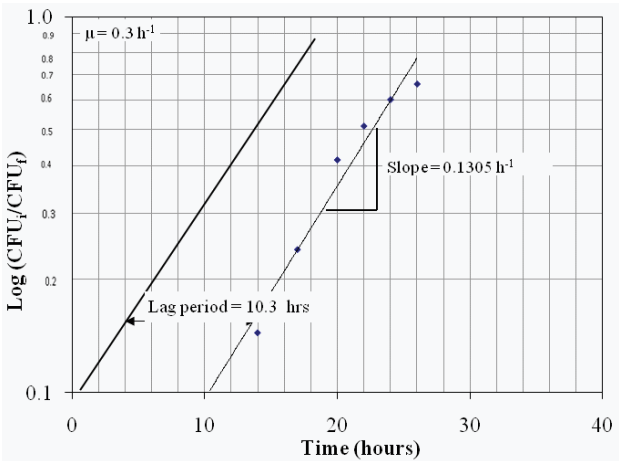


Figure 5: Graphical determination of the lag period and specific growth rate.

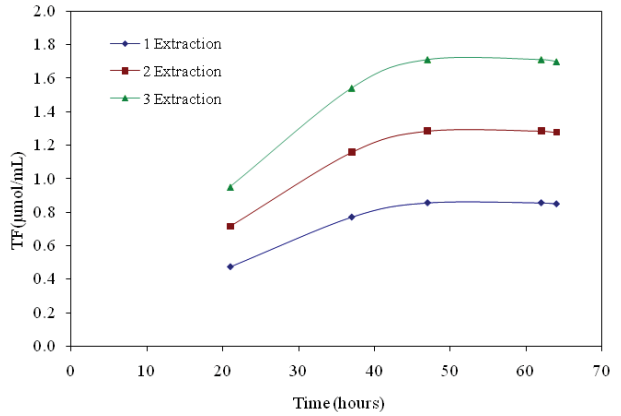


Figure 6: Effect of number of extractions using ethanol on TF yield from *S. venezuelae* (incubation time =1 hour; incubation temperature = 50°C; pH = 6).

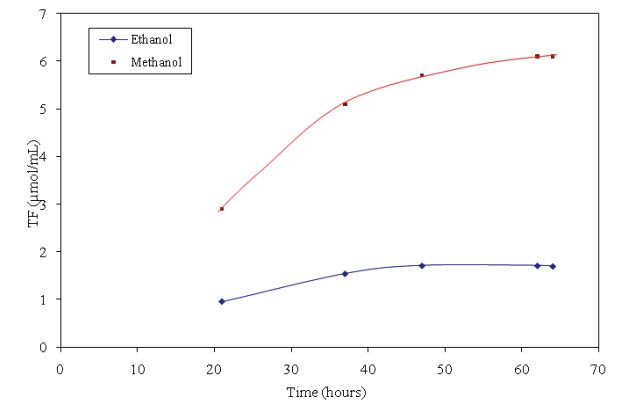


Figure 7: Effect of alcohol type on TF yield from *S. venezuelae* (incubation time = 1 hour; incubation temperature = 50°C; pH = 6; number of extraction =3).

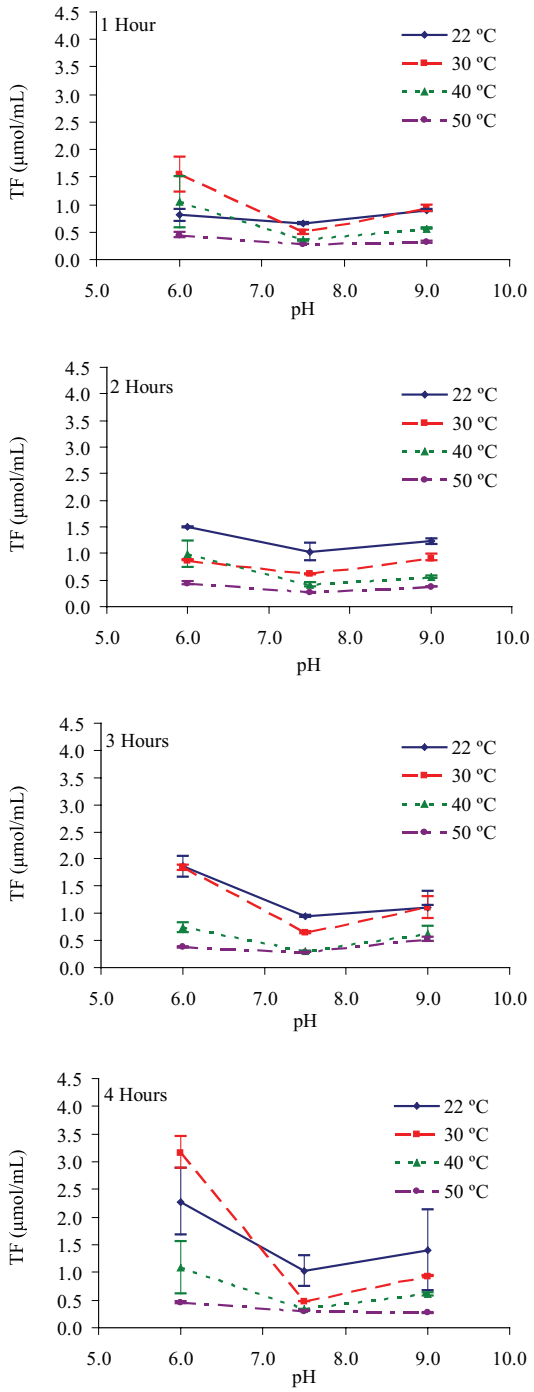


Figure 8: Effect of pH on TF yield at various incubation temperatures.

at different medium pH values and incubation temperatures, respectively. At all incubation times, higher incubation temperatures (40°C and 50°C) resulted in lower TF yields than those obtained at the lower temperatures (22°C and 30°C) but the highest TF yields were always achieved at a pH of 6. It seems that the effect of incubation time was dependent on the temperature. At higher temperatures (40°C and 50°C), 1 hour incubation time produced the higher TF yield while at lower temperature (22°C and 30°C), 4 hours incubation time produced the highest TF yield.

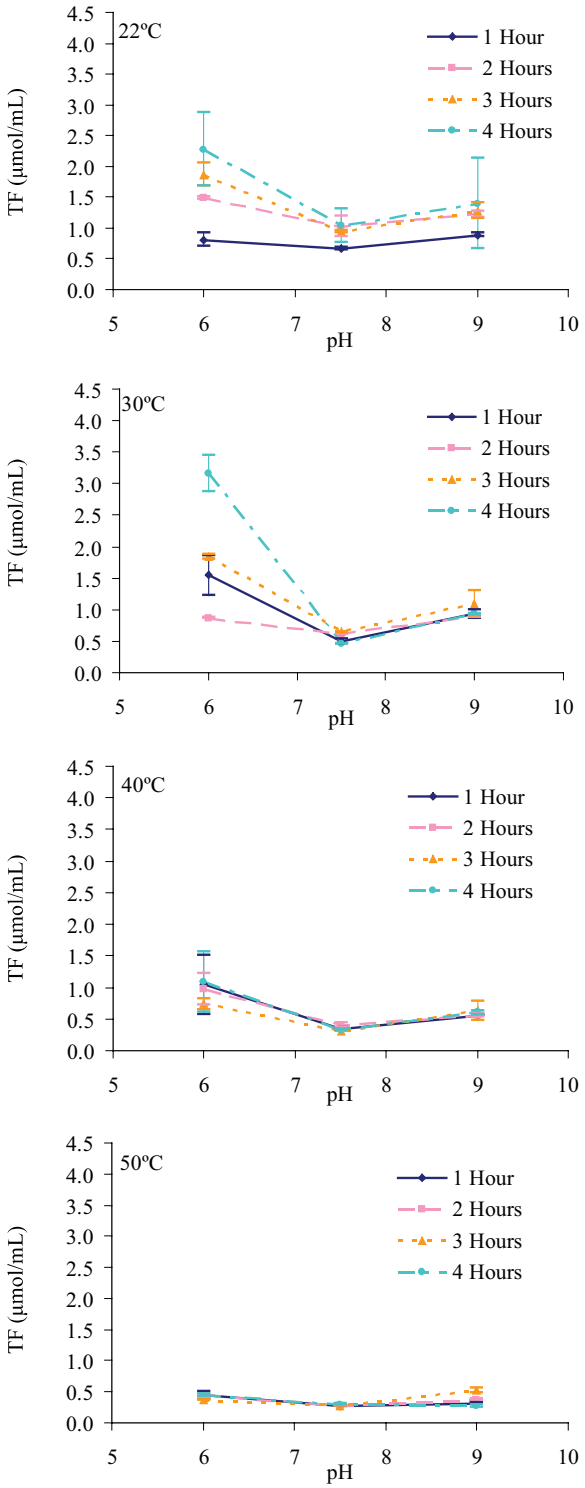


Figure 9: Effect of pH on TF yield at various incubation times.

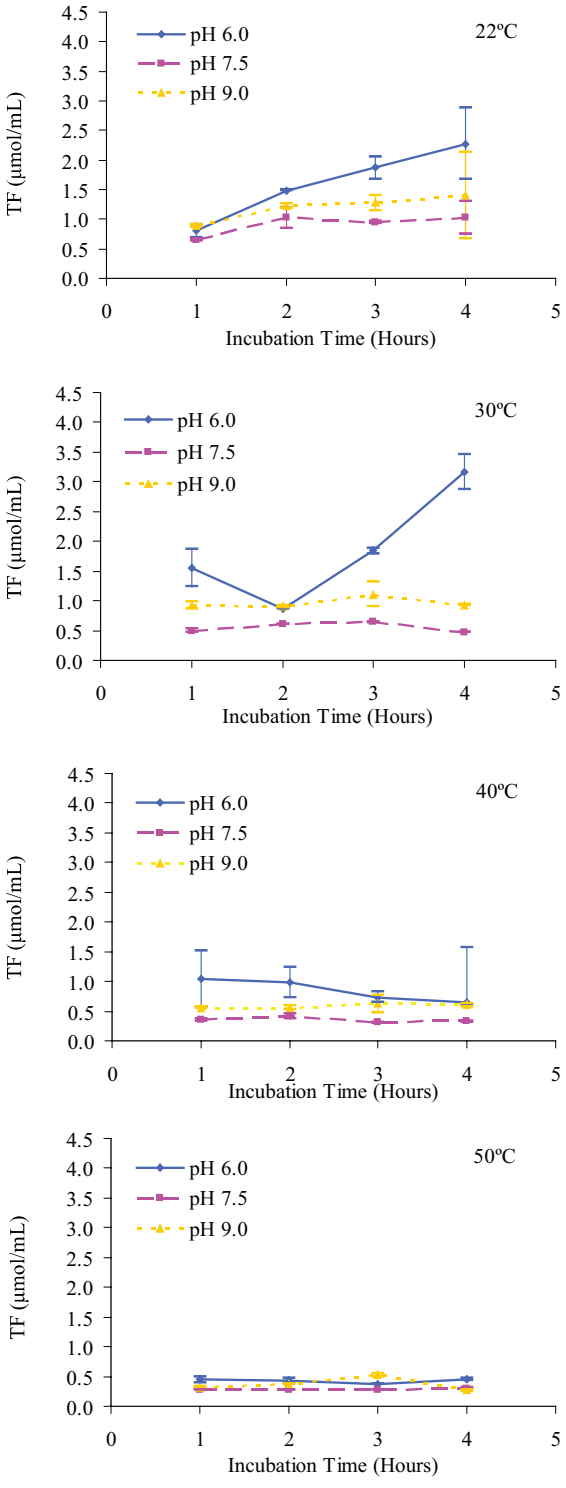


Figure 10: Effect of incubation time on TF yield at various pHs.

Incubation temperature

Figures 12 and 13 show the effect of incubation temperature on TF yield at different pH values and incubation times, respectively. For all incubation temperatures, the highest TF yield was obtained at a pH of 6. However, the incubation temperatures of 22ºC and 30ºC appeared to result in higher TF yields than those observed at higher incubation temperatures (40ºC and 50ºC).

Discussion

Jakeman et al. (2006) monitored *S. venezuelae* population during

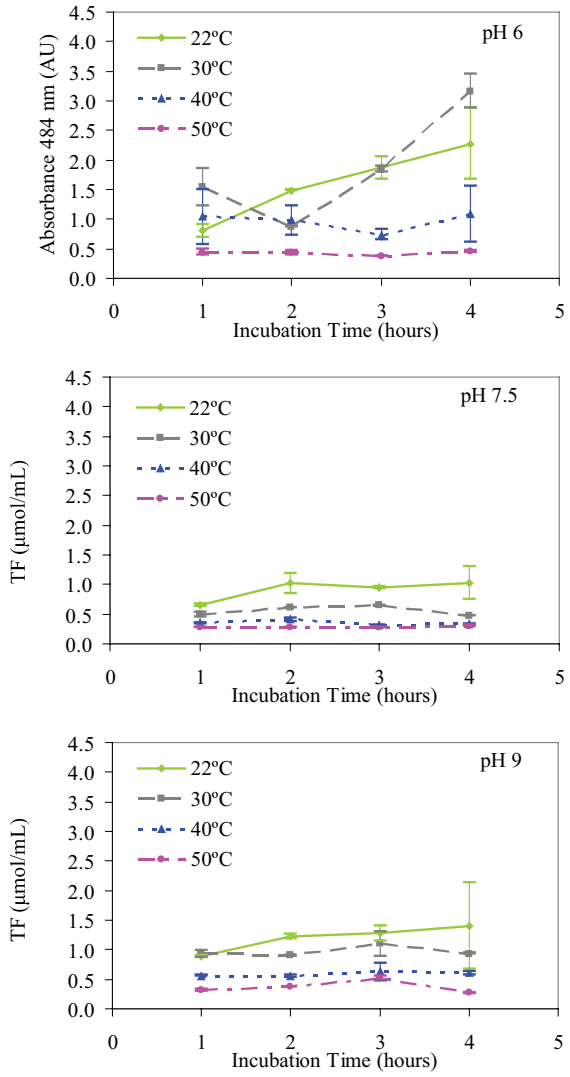


Figure 11: Effect of incubation time on TF yield at various incubation temperatures.

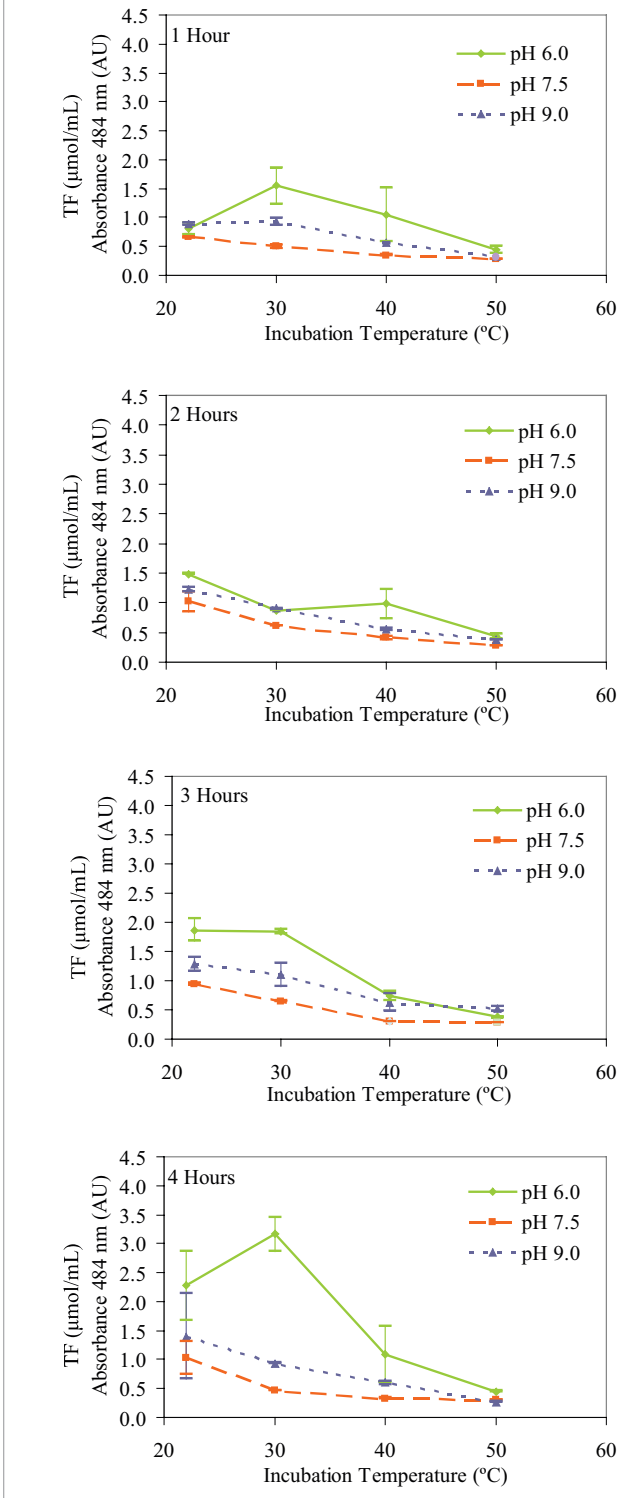


Figure 12: Effect of incubation temperature on TF yield at various pHs.

the growth period by measuring the optical density at 600 nm (OD_{600}). In this study, the change of *S. venezuelae* population during the growth period was monitored by measuring the optical density at 600 nm (OD_{600}), the number of colony forming units (CFU) and the triphenyl formazan yield (TF). The relationships between CFU, OD_{600} and TF are presented in Figure 14. The amount of TF extracted had a much better correlation with CFU than that observed between the CFU and OD_{600}. The results clearly indicated the effectiveness of dehydrogenase activity as an accurate measure of cell growth.

The OD_{600}, TF yield and CFU curves showed a lag period of approximately 10.3 hours, during which *S. venezuelae* adjusted to the new growth medium and environmental conditions. After the initial lag period, the bacteria grew exponentially before reaching the stationary phase at approximately 60 hours. The specific growth measured in this study was 0.3 h^{-1}. [21] reported maximum specific growth rate of 0.23 h^{-1} for *S. venezuelae* grown in media containing soluble starch at 30° C. [22] reported a maximum specific growth rate of 0.14 h^{-1} for *S. venezuelae* grown in MYM medium at 27°C.

The specific TF yield (µmol/CFU) was calculated by dividing the TF yield by the CFU in order to assess the cell activity during the growth period. Figure 15 indicated that the cell activities during the lag phase (0.65 x 10^{-8} µmol/CFU) and stationary phase (0.67 x 10^{-8}µmol/CFU) were lower than that observed during the exponential growth period

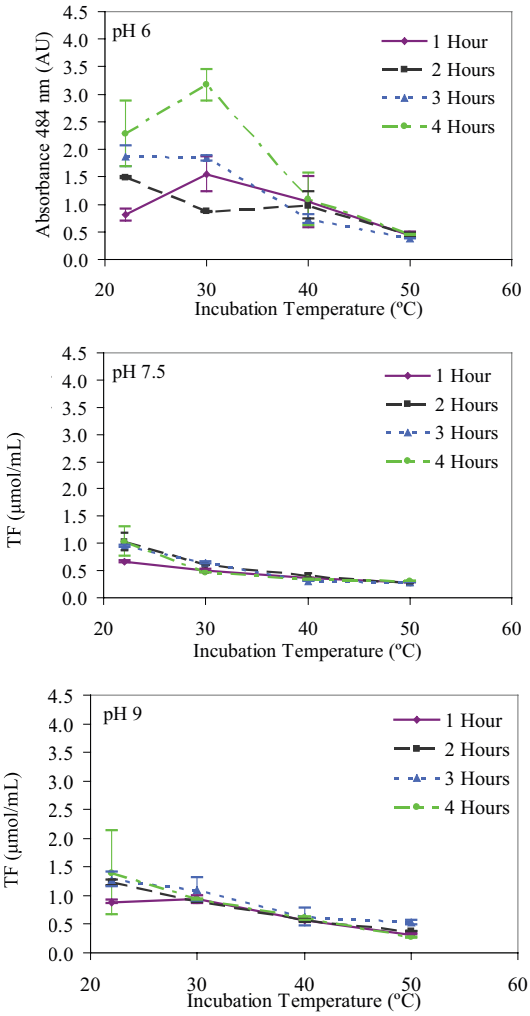
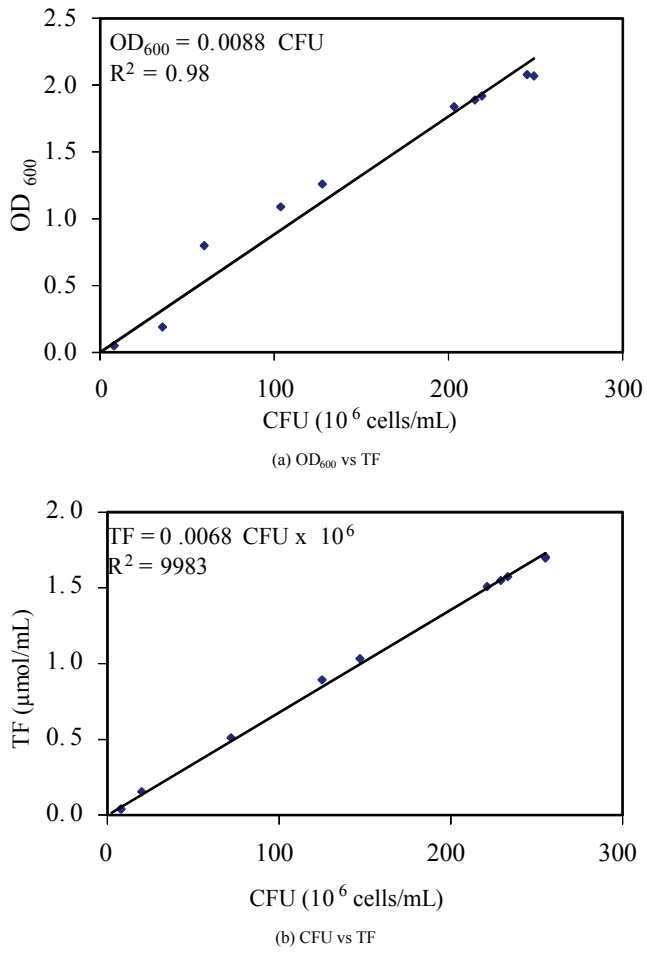


Figure 13: Effect of incubation temperature on TF yield at different incubation times.



(a) OD_{600} vs TF

(b) CFU vs TF

Figure 14: The relationships between CFU, OD600 and TF.

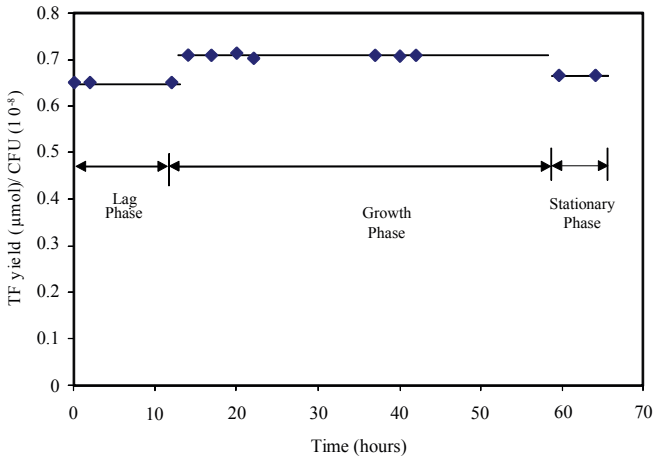


Figure 15: The activity of *S. venezuelae* as measured by specific TF yield during growth in MYM broth.

(0.7 x 10^{-8} µmol/CFU). However, the specific TF yield remained constant during the entire growth period indicating the accuracy of TF as a measure of the cell growth.

Number of extractions

It was observed that for most cases, all of the red colour was removed from the pelletized cells after three extractions. Thus, the dehydrogenase activity test for *S. venezuelae* using TTC should be carried out with at least three extractions in order to achieve the highest TF yield. Other researchers observed a higher recovery of formazan using sequential extractions [17, 23-25]. Green and Nahara (1980) found that extraction with ethanol followed by ethyl acetate achieved the highest yield from muscle cells, but lower yields were observed when the solvents were reversed. They also observed lower TF yields when one and two extractions were done with the same solvent and concluded that the number of extractions was a critical factor that must be considered when developing tests involving solubilization of formazan. In the study by Ghaly and Mahmoud (2006), two extractions with ethanol were found sufficient to extract TF from *Aspergillus niger* vegetative cells. This indicates that the number of extractions may depend on the type of cells.

The specific TF yield (µmol/CFU) of the three extractions from the samples taken at 21, 37, 47, 62 and 64 hours was calculated Table 3. The results indicated that 2nd and 3rd extractions increased the TF yield by

51% and 100% respectively. Also, the cell activity remained constant during the exponential growth period (at the 21, 37 and 47 hours) and slightly decreased during the stationary growth period (after 62 and 64 hours).

Type of solvent

It was noticed that after three washes with ethanol, the cell pellets were still red, which may explain why the TF yield from ethanol was lower. Thus, methanol is the better solvent to use for measuring dehydrogenase activity in *S. venezuelae* using TTC. In the study by Burton and Lanza (1986), the dehydrogenase activities of microbial consortia from sediment slurry samples were measured with TTC using different solvents. They ranked the solvents based on TF yield from highest to lowest as follows: tetrachloroethylene, acetone, propanol, ethanol and methanol. However, the differences between ethanol and methanol were not statistically significant at the 95% confidence level. Tayler and May (2000) [26] found that for the tetrazolium salt INT (2-(4-iodophenyl)-3-(4nitrophenyl)-5-phenyl tetrazolium chloride), methanol was found to be a better solvent than 95% ethanol for the extraction of INT-formazan from bacterial cells. Lee et al. (1988) [27] investigated the extraction of INT-formazan in activated sludge and filamentous bacteria and found methanol to be less effective than dimethylsulfoxide (DMSO) and tetrachloroethylene/acetone as solvents. However, by increasing the permeability of the cells with lysozyme or Triton X-100 prior to using methanol, the same level of formazan yield was achieved as with the other solvents. This indicates that the ability of the solvent to permeate the cells is a critical factor in the extraction of formazan. In the present study, methanol was better able to permeate the cells than ethanol.

The specific TF yield (µmol/CFU) after three extractions with ethanol and methanol at 21, 37, 47, 62 and 64 hours was calculated Table 4. The results showed that the TF yield for methanol was 259 % higher than that of ethanol. Also, the specific TF yield remained constant during the exponential growth period (at the 21, 37 and 47 hours) and slightly decreased during the stationary growth periods (at the 62 and 64 hours).

Medium pH

Several researchers reported high TF yields at different pH values. For example, Mahmoud and Ghaly (2004) found that at pH less than 7, no reduction of TTC occurred for both cheese whey (*Kluyveromyces fragilis*) and compost materials (mixed culture). Ghaly and Ben-Hassan (1993) [28] found that maximum dehydrogenase activities for both *Kluyveromyces fragilis* and *Candida pseudotropicalis* yeasts grown in cheese whey were at a pH of 7 and the activities were reduced at the acidic and basic levels of pH. Backor and Fahselt (2005) [29] reported that significantly acidic pHs (1.5 - 3) resulted in lower TTC reduction in lichens. Ghaly and Mahmoud (2006) [30] observed higher TF yield at a pH of 9 for *A. niger* grown in chitin. However, Mahmoud and Ghaly (2004) reported non-enzymatic reduction of TTC to TF at high pH values. In this study, a pH of 6 was the most appropriate value for measuring dehydrogenase activity in *S. venezuelae* during growth in MYM broth. It is not clear however if non-enzymatic reduction of TTC to TF occurred at pH 9.

Incubation time

The results showed slight increase in TF yield as when the incubation time increased from 1 hour to 4 hours. Several investigators reported that incubating samples for longer times increased the extent of TTC reduction to TF. Mahmoud and Ghaly (2006) reported that TF yield for *A. niger* grown in chitin increased exponentially when incubation time was increased form 1.5 hours to 4.5 hours. Ghaly and Ben-Hassan (1993) reported increased TF yield with increased incubation time for both *Kluyveromyces fragilis* and *Candida pseudotropicalis* yeasts grown in cheese whey, but the TF yield started to plateau after 80 hours in both cases. Mathew and Obbard (2001) [31] reported increased INT-formazan yield with increased incubation time for petroleum-contaminated beach sediments, but the TF yield started to level off after 22 hours of incubation. Although the TF yield obtained at an incubation period of 4 hours was slightly higher than the TF yield obtained at an incubation time of 1 hour, it is more practical to use 1 hour since the resulting TF yield is measurable and can provide good representation of cell growth and activity.

Time (hours)	Specific TF Yield (10^{-8} µmol/CFU)		
	1st Extraction	2nd Extraction	3rd Extraction
21	0.35	0.53	0.70
37	0.35	0.52	0.70
47	0.35	0.53	0.70
62	0.33	0.50	0.67
64	0.33	0.50	0.67

Table 3: Specific TF yield for various ethanol extractions at various sampling time

Time (hours)	Specific TF Yield (10-8 µmol/CFU)	
	Ethanol	Methanol
21	0.70	2.51
37	0.70	2.51
47	0.70	2.51
62	0.67	2.49
64	0.67	2.49

Table 4: Specific TF yield for ethanol and methanol at various sampling time.

Incubation temperature

In the literature, there have been reports of higher TF yields as a result of increasing incubation temperature for microbial populations [30,32]. In this study, higher temperature (40°C and 50°C) showed a negative effect on the activity of the bacteria probably due to enzymatic inhibition at higher temperature [34]. According to Breed (1957) [33] *S. venezuelae* are soil bacteria, and therefore, achieve optimal growth with the lower temperature ranges investigated (22°C-30°C) in this study. Doull et al. (1993) reported a decreased growth for *S. venezuelae* at temperatures of 37° C and 42° C, compared to that at a control temperature of 27° C. Therefore, 30° C is an optimum temperature for measuring the dehydrogenase activity of *S. venezuelae* with TTC test.

Conclusions

A dehydrogenase activity measurement test using triphenyl tetrazolium chloride (TTC) was successfully developed for *Streptomyces venezuelae* growth in MYM broth. TF yield (µmol/mL) was related to the number of cells was measured by optical density (OD_{600}). It was found that methanol was able to extract a greater yield of the red triphenyl formazan (TF) than ethanol and that the TF yield increased with the number of extractions. High TF yields were observed at low pH value and/or low temperatures. Lower temperatures (22-30°C) required longer incubation time compared to higher temperature (40-50° C). Based on the results obtained from this study, the optimum conditions for measuring the dehydrogenase activity of *S. venezuelae* (reducing TTC to TF and extracting highest amount of TF) are three extractions with

methanol after incubation time of 1 hour at a medium pH of 6 and incubation temperature of 30°C.

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