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Evaluating the Concept of Reducing Sugar (Glucose) and Growth in L-Asparaginase Production Using Some Selected Marine Actinomycetes

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ABSTRACT: Reducing sugar is a sugar that serves as a reducing agent. It falls under the category of carbohydrate or natural sugar but it consist of either a free aldehyde group (-CHO) or a ketone groups called the reducing end of the sugar. Microbial growth describes an increase in cell number of microorganisms provided with suitable nutritional and environmental conditions. This study evaluated the concept of reducing sugar and growth in L- asparaginase production using some selected marine actinomycetes. Different isolates of marine actinomycetes were screened for L- asparaginase production using rapid plate method and secondary screening was carried out by submerged fermentation using inoculum size of 5 % v/v, the inoculated. Czapek Dox broth was incubated at 30 ± 2 º C using a water bath shaker for 7 days at 120 rpm. Production of L -asparaginase was carried out using Czapek Dox broth supplemented with L-asparagine (1 g/L), as production medium that was incubated at 30 ± 2 ºC for 7 days at 120 rpm. Optical density (growth rate) and reducing sugar concentration were determined spectrometrically at 600 and 540 nm, respectively. *Actinosynnema mirum* had the highest optical density of 1.965 ± 0.001 and *Streptomyces coelicolor* had the least optical density of 1.321±0.004. *Streptomyces nobilis* reached death phase on day 5 of production. The reducing sugar concentration reduced from 47.88±0.16 mg/ml to 6.77 ± 0.06 mg/ml for *Actinosynnema mirum*. Conclusively, appreciable amount of glucose concentration, reduced from day 0 to day 7 and growth of isolates in the course of production of L- Asparaginase followed a normal growth pattern, with Lag phase, growth phase, stationary phase and death phase.

Keywords: Aldehyde, Ketone, Cell number, L-asparagine, Spectrometrically

Introduction

A reducing sugar is a sugar that serves as a reducing agent. It falls under the category of carbohydrate or natural sugar but it consist of either a free aldehyde group (-CHO) or a ketone groups called the reducing end of the sugar. They act as reducing agent by effectively donating electrons to some other molecules through oxidation. Some disaccharides, oligosaccharides, polysaccharide and all monosaccharide are reducing sugars. The most common example of ketose is fructose whereas glucose and galactose are aldoses. The most common examples of non-reducing sugar is sucrose because it has two unit of monosaccharide's that are held together very tightly by a glycosidic linkages between the C-2 carbon of the fructose and the C-1 of glucose (Vicchia *et al.,* 2013).

Microbial growth describes an increase in cell number. Provided with the right conditions, (food, temperature, pH, water availability/activity) microbes can multiply very quickly. While growth for multicellular organisms is typically measured in terms of increase in size of a single organism, microbial (marine actinomycetes) growth can be measured by the increase in population either by measuring the increase in cell number or the increase in overall cell mass.

Marine actinomycetes just like all bacteria and archaea reproduce asexually. They engage in binary fission where a single cell splits into two equal cells. Actinomycetes are known to produce spores. The process begins with cell enlongation, which requires careful enlargement of the cell membrane and cell wall in addition. Actinomycetes are easy to grow in the laboratory for the production of different metabolites. In a typical batch culture, bacteria will grow in a predictable pattern, resulting in a growth curve composed of four distinct phases. The lag phase, the exponential or log phase, the stationary phase and the death phase (Mishra and Das, 2002).

The production of secondary metabolites (L-asparaginase) in marine actinomycetes is greatly influenced by various fermentation parameters such as available nutrients, pH, temperature, agitation, mineral salts, metal ions, precursors, inducers and inhibitors. pH is the most essential parameters control in enzyme secretions because microbes are sensitive to the concentrations of hydrogen ions present in the fermentation process (Bibb, 2005; Geshava *et al.*, 2005). According to Zia *et al.* (2013), addition of glucose during static incubation medium, would lead to depressed cell growth and L-asparaginase production. Also, yields of extracellular L- asparaginase is achieved with the addition of glucose followed by sucrose and fructose, but starch repressed L-asparaginase production. Understanding how cells make decisions and why they make the decisions that they make is of fundamental interest in system biology. In this study, we looked at the concept of reducing sugar and growth in L- asparaginase production using some selected marine actinomycetes.

Materials and Methods

Isolation/primary screening of marine microbial/actinomycetes isolates for asparaginase production: Isolation of marine actinomycetes was carried out by pour plate using Starch casein agar. Inoculated plates were incubated at 30 ± 2 °C for 4-5 days and pure cultures were obtained by streaking. Different isolates of marine actinomycetes were screened for asparaginase production using rapid plate method according to Gulati *et al.* (1997). Modified M medium was used, and it contained (per 1000 ml of distilled water): $Na_2HPO_4.2H_2O$ 6.0 g, KH_2PO_4 3.0 g, NaCl 0.5 g, L –asparaginase 5.0 g, 1 mol L^{-1} $MgSO_4.7H_2O$ 2.0 ml, 0.1 mol L^{-1} $CaCL_2.2H_2O$, 1.0 ml, 20 % glucose stock 10.0 ml, agar 20.0 g/l.

The medium was supplemented with 0.009 % of phenol red that was initially prepared with absolute ethanol (2.5 g in 97 ml of ethanol, pH adjusted to 7.0 using NaoH). The pH of the medium was adjusted to 6.8. Control plates were prepared without L- asparagine and Phenol red. The medium was autoclaved at 121 ^{0}C for 15 mins, it was allowed to cool and poured aseptically on different plates. Inoculation was carried out using 96 hrs old pure plate cultures of actinomycetes. The plates were incubated at 37 °C for 4-5 days and zones of clarity were measured.

Secondary screening (submerged fermentation): Secondary screening was carried out according to Senthil and Selvem, (2011). Inoculation was carried out aseptically using inoculum size of 5 % (2.5 ml into 50 ml of broth) containing spore count of 2.5 x 10^5 cells/ml. The inoculated broth was incubated at 30 ± 2 °C using a water bath shaker for 7 days at 120 rpm.

Preparation of inoculum for enzyme production (screening): Inoculum for enzyme production was prepared by inoculating starch casein broth with pure culture of marine actinomycetes. The broth was then incubated for 5-6 days.

Production of L-asparaginase: Production of L -asparaginase was carried out in Erlenmeyer flask containing Czapek Dox broth supplemented with L-asparagine (1g/L), as production medium for 7 days at 120 rpm and at 28 °C. A 5 % inoculum was used containing 2.5x 10^5 cells/ml. During production; about 5 ml of the broth was withdrawn at intervals to determine the optical density, reducing sugar concentration and L-asparaginase activity spectrometrically (Hanif, 2017).

Determination of optical density: Using cuvette, optical density was used to measure the turbidity of the withdrawn broth at 600 nm at different time intervals.

Determination of reducing sugar concentration: During the production of L- Asparaginase, reducing sugar was determined using 3, 5-dinitrosalicyclic (DNSA) method. It detects the presences of free carbonyl group of reducing sugars. 1ml of the supernatant was taken and dispensed into a sterile test tube and 100 µl of DNSA reagent was added, the solution was boiled for 5 mins and then cooled. 2 ml of distilled water was then added and the colour was allowed to stabilize. It was then read at 540 nm using Spectrophotometer (Gusakov *et al.*, 2011)

$$\text{Amount of reducing sugar (mg/ml)} = \frac{\text{Absorbance}}{\text{Slope}}$$

Where Y = Absorbance,
Slope = intercept (line of best fit).

Determination of glucose standard curve: A gram of glucose was dissolved in 99 ml of sterile distilled water, and it was assay for the presence of free carbonyl group using different concentrations. 0, 20, 40, 60, 80, 100 µl of the prepared stock. 100µl of 3, 5-dinitrosalicyclic (DNSA) reagent was added to the varied concentrations of glucose and then boiled for 5 mins and cooled. 2 ml of distilled water was then added and the colour was allowed to stabilize. It was then read at 540 nm using spectrophotometer. The absorbance reading was plotted against the different concentrations to get the line of best fit.

Determination of L-asparaginase activity: Enzyme assay was carried out for every 24 hr interval. A 5 ml of the production medium was withdrawn using a sterile pipette and carefully dispensed into a bottle using cuvette, the optical density was checked spectrophotometrically at 600 nm. The sample was then centrifuged at 120,000 revolution /minute. The supernatant was then used for the assay. 0.1 ml enzyme solution was added to 0.9 ml buffer (pH 8.5) and 1 ml of the amino acid L-asparagine. It was incubated at 37 °C for 10 min, thereafter, 0.5 ml of TCA was added to stop the reaction and precipitate the different protein molecule. The sample was then centrifuged thereafter, 50 µl of supernatant was collected and placed in a clean tube. 2000 µl of distilled water was added to the supernatant with 250 µl of Nessler's reagent and 250 µl NaoH solutions were added and the solution was left for 15 min. Using the spectrophotometer, the result was read at 600 nm. Ammonia standard curve was prepared. L-asparaginase activity was calculated with the formula below (Luhana *et al.*, 2013).

$$\text{Activity of L-asparaginase} = \frac{\text{Absorbance}}{\text{Slope of graph X Time of incubation X Volume of protein}} \text{x DF}$$

Results

The results of optical density of marine actinomycetes in L-asparaginase production are presented in Table 1. *Actinosynnema mirum* (isolate P), had the highest optical density of 1.965 ± 0.001 and *Streptomyces coelicolor* had the least optical density of 1.321±0.004. Isolate U (*Rhodococcus triatomae*) had the highest optical density of 1.400 ± 0.005 on day 2 of production and isolate V (*Streptomyces coelicolor*) had the least optical density of 0.701±0.001. There were no significant difference on results obtained from day 0. *Streptomyces nobilis* (isolate L) reached death phase on day 5 of production (Fig. 1).

Table 1: Optical density of marine Actinomycetes during production (600 nm)

Isolates	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
L	0.031^{a}±0.0005	0.304^{a}±0.0005	1.191^{b}±0.001	1.569^{b}±0.0005	1.559^{b}±0.0005	1.569^{b}±0.0005	1.191^{a}±0.001	0.059^{a}±0.0005
P	0.02^{a}± 0.0005	0.051^{a}±0.006	0.854^{a}±0.0005	1.452^{a}±0.157	1.897^{b}±0.001	1.903^{b}± 0.002	1.965^{b}±0.001	1.82^{c}±0.002
U	0.036^{a}±0.0005	0.372^{a}±0.036	1.400^{b}±0.0005	1.735^{b}±0.0005	1.809^{b}±0.001	1.927^{b}±0.001	1.855^{b}±0.001	1.844^{c}±0.002
S	0.034^{a}±0.002	0.043^{a}±0.0005	0.925^{a}± 0.001	1.336^{a}±0.001	1.456^{a}±0.0005	1.845^{b}±0.002	1.849^{b}±0.061	1.83^{c}±0.002
V	0.034^{a}±0.001	0.268^{a}±0.009	0.701^{a}±0.001	1.286^{a}±0.001	1.32^{a}±0.011	1.321^{a}±0.0005	1.332^{a}±0.006	1.175^{b}±0.002

Values are mean ± S.D; n=3. Mean values with similar superscripts within each column are not significantly different, P>0.05.

Note:
Isolates
P -------- *Actinosynnema mirum*
V---------*Streptomyces coelicolor*
U----------*Rhodococcus triatomae*
S--------- *Streptomyces avermitilis*
L-------- *Streptomyces nobilis*

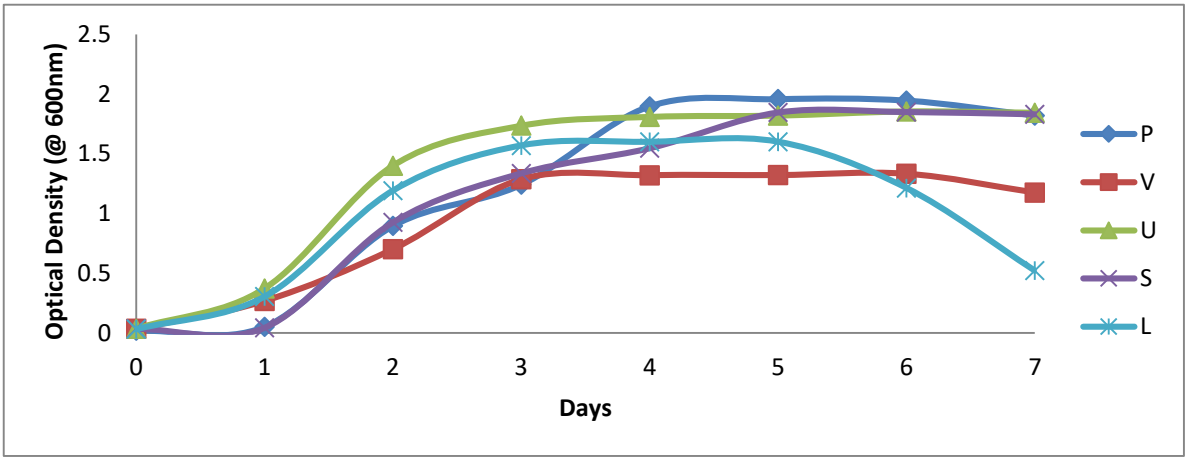

Figure 1: Optical density of selected marine actinomycetes in L-asparaginase production

The results of the evaluation of reducing sugar concentrations in L. Asparaginase production are presented in Table 2. The reducing sugar concentration reduced from 47.88±0.16 mg/ml to 6.77 ± 0.06 mg/ml for *Actinosynnema mirum* (isolate P) showing therefore, that the organism was able to utilize 41.11 mg/ml of glucose to carry out its metabolic activities. *Rhodococcus triatomae* (isolate U) showed a reduction from 57.11±0.16 to 18.88±0.06 mg/ml on day 7 of production. *Streptomyces coelicolor*, had an initial concentration of 49.22 ± 0.064 on day 0 and on day 7, the concentration of reducing sugar reduced to 22.55 ± 0.16 mg/ml indicating that the isolate utilized 26.67 mg/ml in carryout its metabolic activity (Fig. 2).

Table 2: Determination of glucose concentration during enzyme production (mg/ml)

Isolates	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
L	53.77^{b}±0.16	52.88^{c}±0.16	47.22^{b}±0.06	40.0^{b}±0.00	33.88^{ab}±0.06	26.88^{ab}±0.06	23.55^{b}±0.06	20.55^{b}±0.06
P	47.88^{a}±0.16	41.22^{a}±0.16	37.77^{a}±0.64	29.44^{a}±0.33	21.88^{a}±0.06	17.22^{a}±0.16	6.77^{a}±0.06	0.55^{a}±0.16
U	57.11^{c}±0.16	51.11^{b}±0.16	46.77^{b}±0.16	36.33^{ab}±0.51	33.00^{ab}±0.00	32.66^{b}±0.00	27.88^{c}±0.06	18.88^{ab}±0.06
S	52.66^{b}±0.00	51.88^{b}±0.16	51.33^{c}±0.00	40.55^{b}±0.06	38.22^{b}±0.16	29.22^{ab}±0.06	22.00^{b}±0.00	21.22^{b}±0.06
V	49.22^{ab}±0.064	47.77^{ab}±0.06	46.77^{b}±0.06	43.33^{c}±0.00	40.55^{c}±0.06	35.77^{c}±0.06	23.88^{b}±0.06	22.55^{b}±0.16

Values are Mean ± S.D; n=3. Mean values with similar superscripts within each column are not significantly different, P>0.05.

Note:
Isolates
P -------- *Actinosynnema mirum*
V---------*Streptomyces coelicolor*
U----------*Rhodococcus triatomae*
S--------- *Streptomyces avermitilis*
L-------- *Streptomyces nobilis*

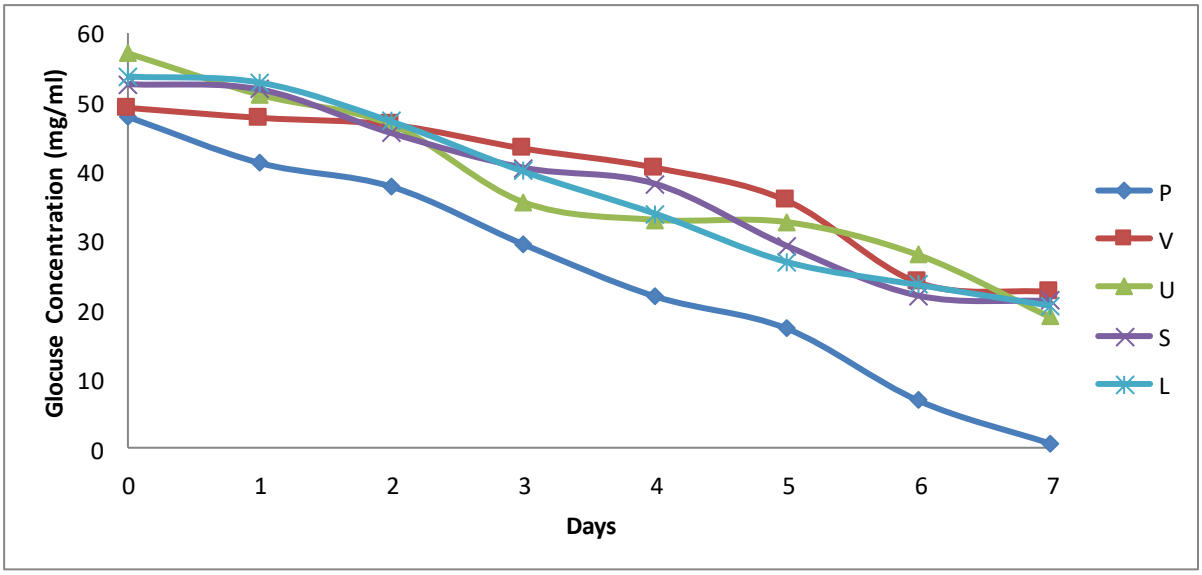

Figure 2: Reducing sugar (glucose) concentration of marine actinomycetes in L-asparaginase production using modified Czepek Dox medium.

Table 3 explains the determination of L-asparaginase activity, with *Streptomyces nobilis* (isolate L) having the least enzyme activity of 12.01±0.22 µmol/min/ml of L-asparaginase activity, while *Streptomyces avermitilis* and *Rhodococcus triatomae* had highest values of 20.99±0.58 and 20.21 ± 0.58 µmol/min/ml L-asparaginase respectively on day 2 of production. Isolate U also had highest enzyme activity of 10.34±0.43 µmol/min/ml on day 4 of production. Mean values with similar superscripts within each column are not significantly different, P>0.05. This showed that there were no significant difference in L-asparaginase activity for all the isolate on day 0 (Fig. 3).

Table 3 : Determination of L-Asparaginase activity (µmol/min/ml) during production

Isolates	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
L	0.293^{a}±0.011	1.89^{b}±0.01	12.01^{a}±0.22	9.08^{b}±0.17	3.45^{ab}±0.01	0.72^{a}±0.03	1.20^{a}±0.19	0.55^{a}±0.12
P	0.073^{a}±0.011	3.12^{c}±0.24	15.68^{ab}±0.04	3.32^{a}±0.02	2.43^{a}±0.04	1.46^{b}±0.04	1.7^{ab}±0.03	0.86^{a}±0.01
S	0.07^{a}±0.011	1.94^{b}±0.58	20.99^{c}±0.58	6.21^{ab}±0.18	3.54^{ab}±0.94	2.68^{ab}±0.72	3.76^{b}±0.74	1.46^{b}±0.22
U	0.23^{a}±0.011	1.68^{a}±0.11	20.21^{c}±0.58	13.66^{c}±0.04	10.34^{c}±0.43	9.08^{c}±0.05	4.30^{c}±0.27	1.62^{b}±0.32
V	0.03^{a}±0.011	1.58^{a}±0.03	17.66^{b}±0.38	4.22^{a}±0.09	9.1^{b}±0.02	5.72^{b}±0.02	4.39^{c}±0.10	3.43^{c}±0.82

Values are mean ± S.D; n=3. Mean values with similar superscripts within each column are not significantly different, P>0.05.

Note:
Isolates
P -------- *Actinosynnema mirum*
V---------*Streptomyces coelicolor*
U----------*Rhodococcus triatomae*
S--------- *Streptomyces avermitilis*
L-------- *Streptomyces nobilis*

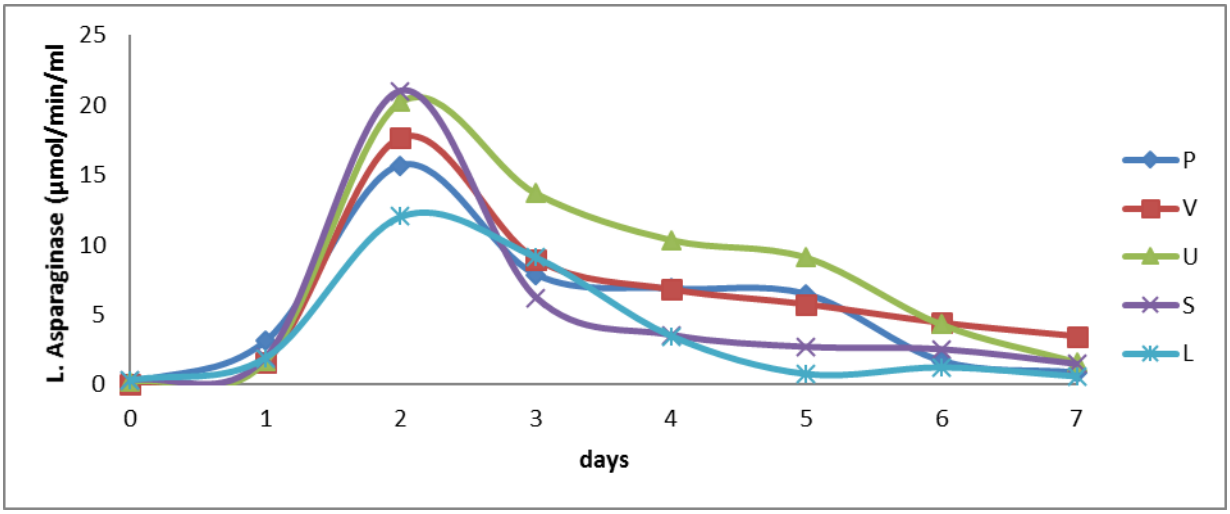

Figure 3: Determination of L-asparaginase activity using selected marine actinomycetes

Discussion

The growth of isolates in the course of production of this enzyme followed a normal growth pattern, with Lag phase, growth phase, stationary phase and death phase. It was observed that isolate S (*Streptomyces avermitilis*) and P (*Actinosynnema mirum*) maintained a reasonable level of Lag phase where by the organism tend to adjust to environmental factor and to the medium. Comparing the different growth levels, *(Actinosynnema mirum* and (*Streptomyces avermitilis*) also had the highest growth level isolate V *(Streptomyces coelicolor)* reached stationary phase on day 3 and a decline /death phase on day 6-7. At stationary phase, the growth rate and death rate are equal. Stationary phase is the phase at which growth ceases, but cells remains metabolically active, and also the proteins synthesize at this phase are indispensible as they confer viability to the organisms.

However, reducing sugar concentration was measured alongside using the 3, 5-DNSA method. This sugar is a sugar that can be oxidized by mild oxidizing agent. Examples of oxidizing agents are oxygen (0_2), ozone (0_3), hydrogen peroxide (H_2O_2) and other inorganic peroxides, Fenton's reagents, fluorine (F_2), chlorine (Cl_2) and other halogens, nitric acid (HNO_3) and nitrate compounds, sulfuric acid (H_2SO_4), peroxymonosulfuric acid (H_2SO_5). Oxidizing materials are liquid or solids that readily give off oxygen or other oxidizing substance. Aluminium nitrate, ammonium persulfate, Barium perioxide, hydrogen peroxide solutions are examples of strong oxidizing agents. In the Czepek-Dox medium used, it contained sodium nitrate ($NaNO_3$), this acts as the oxidizing agents to reduce glucose to two molecules of pyruvate (Gerhart, 2009).

However during microbial growth, fermentation occurs, a process in which glucose undergoes glycolysis, to produce two molecules of pyruvate, which is later converted to two molecules of lactic acid. When pyruvate is produced, two ATP (net and two NADH is produced. Nicotinamide adenine dinucleotide hydrogen (NADH) is oxidized and a pyruvate is reduced. At the end of fermentation, the concentration of lactic may increase and this may diffuse into the microbial cell. Accumulation of excessive lactic acid could lead to toxicity of the medium, this invariably would cause cell shrinking and finally death may occur. However, the presence of lactic acid in a medium would lead to a fall in the pH value. That is why a reduction in glucose concentration leads to stationary phase and finally death phase, because at the end of fermentation there is accumulation of lactic acid which makes the medium acidic.

In this study, appreciable amount of glucose concentration, reduced from Day 0 to Day 7. In the cause of producing L-asparaginase using isolate L, the concentration reduced from 53.77± 0.16 mg/ml to 20.55 ± 0.064 mg/ml, for isolate V, the reduction was from 49.22 ± 0.06 to 22.55± 0.16 mg/ml. Considering the optical density, isolate P had absorbance reading of 1.965 ± 0.001 at 600 nm on Day 6. Its reducing sugar concentration reduced from 47.88 ± 0.16 mg/ml to 6.77 ± 0.06 mg/ml; it means therefore that the organism was able to utilize 41.11 mg/ml of glucose to carry out its metabolic activities.

However, some rare marine Actinomycetes require sea water for their growth (Jensen *et al.*, 2005b). The newly describe genera *Salinospora*, *Marinispora* both of which requires sea water for their growth and have marine chemotype signature and *Aeromicrobium murium* which also has an obligate requirement for salt. Another recently

characterized genus, *Salinibacterium* can tolerate up to 10 % NaCl, but does not have a salt requirement for growth (Han *et al.,* 2003*).*

According to Gulati 1999, the plate study is advantageous as the method is quick and L-asparaginase production visualized directly from the plates. Generally, the isolate screened had various degrees of clarity of zones. However, some isolate had late /slow L-asparaginase production capacity hence submerged fermentation process was used for the secondary screening. Jha *et al.,* (2012) stated that the most adopted method of production of L-asparaginase enzyme is by submerged fermentation and it has been adopted throughout the world. Some limitation of this process was observed later and to overcome those disadvantages, solid state fermentation process has been adopted (Mishra and Das, 2002.) The method has been said to have the following advantages over submerged fermentation; higher product yield, low capital cost , low energy input, simple fermentation media and low moisture requirement.

However, in this study Isolate L *(Streptomyces nobilis)* maintained a gradual growth level from Day 0 to Day 3 and a short stationary phase. Day 5 marked the beginning of the death phase. L-asparaginase activity of the different isolate increase from Day 0 to Day 2, meaning that the organisms were able to utilize the amino acid during the exponential phase. The peak of this enzyme activity was on day 2 for all the marine actinomycetes, *Streptomyces avermitilis* had the highest value of 20.99 ± 0.58 µmol/min/ml and the lowest being isolate L. A unit of L-asparaginase is the amount of enzyme that can breakdown or catalyze 1µmole of L-asparagine to aspartic acid and ammonia

Meanwhile, the production of significant value of enzyme activity on growth/exponential phase makes the enzyme production a primary metabolite. These metabolites are mainly synthesized when there is nutrient availability to the cell. Shanthipriya *et al.* (2015) studied three thermophilic coprophilous fungi (*Thielavia terrestris, Malbranchea cinnamomea* and *Scytalidium thermophilum*) and discovered that the maximum L-asparaginase production was recorded in a glucose medium supplemented with 0.5 % L-asparagine monohydrate on 8th day of incubation. This means that the enzyme is most likely to be produced at the stationary phase, where there is exhaustion of nutrient hence a secondary metabolite. In this study, *Streptomyces nobilis* had the least enzyme activity of 12.01±0.22 µmol/min/ml of L-asparaginase activity, while *Streptomyces avermitilis* and *Rhodococcus triatomae* had highest values of 20.99±0.58 and 20.21 ± 0.58 µmol/min/ml L-asparaginase respectively.

Conclusion

The production of many secondary metabolite including antibiotics, L-asparaginase is coupled with morphological differentiation. L -asparaginase can be secreted either as primary or secondary metabolites. Increase in cell mass of the different marine actinomycetes followed a normal growth pattern and reducing sugar concentration reduced as the organisms increased in cell number. Indeed, researchers have observed a greater production of secondary metabolite during the transition from vegetative growth to aerial growth.

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