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Bioproduction of alpha-amylase from *Bacillus amyloliquefasciens* using taro (*Colocasia esculenta*) as a medium

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ABSTRACT

Amylases are among the most important industrial enzymes, with diverse applications ranging from conversion of starch into sugar syrups to the production of cyclodextrins for the pharmaceutical industry. *Bacillus amyloliquefaciens* NBRC 15535 can be a potential source of amylase production. The use of taro (*Calocasia esculenta*) as primary starch submerged for α-amylase production and scale up contributes as substrate for industrial enzyme production. Report on taro as fermentation substrate are scarce despite its high starch content and availability in tropical regions like Philippines. This study aimed to optimize alpha-amylase production conditions for large-scale industrial applications. Stat-Ease Design-Expert software was employed to design the bioproduction parameters, specifically pH, temperature, and substrate concentration. For the scale-up process, a two-level factorial design was utilized to determine the optimal fermentation conditions. Based on small-scale experimental results, the optimum conditions were identified as pH 8.0, 50°C, and a 4% substrate concentration.

Statistical analysis demonstrated that temperature was the only significant factor influencing α-amylase production ($p < 0.0001$), while pH, substrate concentration, and their interactions were not significant. Scale-up experiments revealed that conditions favoring biomass accumulation did not necessarily correspond to maximal specific enzyme activity, highlighting the importance of distinguishing cell growth from enzyme production. Time-course analyses indicated that the culture remained in the exponential growth phase within 24 h of fermentation, suggesting that the true optimum for α-amylase production was not yet reached

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under the evaluated conditions. Therefore, extending the incubation period to 48–72 h and exploring broader pH ranges are proposed to capture potential growth-associated or stability-driven increases in enzyme yield. Overall, this study establishes baseline process parameters and demonstrates the feasibility of taro-based media for scalable α-amylase production to meet industry demand, while providing a clear framework for future optimization.

Keywords: amylase, *Bacillus amyloliquefaciens*, *Colocasia esculenta*, RSM

INTRODUCTION

Alpha-amylase is a calcium-dependent enzyme that is highly important in the catalysis of starch (Gangadharan *et al*., 2009; Xian *et al*., 2015). Generally, it is an endoamylase which cleaves through hydrolysis the α, 1–4 glycosidic bonds located in the inner (endo) part of the amylose or amylopectin chain, causing the formation of both linear and branched oligosaccharides with different lengths but with the same α-configuration (van der Maarel *et al*., 2002; Gupta *et al*., 2003; Xian *et al*., 2015). This particular type of enzyme which constitutes a highly diverse group of glycosyl hydrolases shares several characteristics common with other groups of amylases (exoamylases, debranching enzymes and transferases) including the elucidation of a (β/α) 8-barrel structure or the triose-phosphate isomerase (TIM) structure hydrolysis or formation of glycosidic bonds in the α conformation and the presence of several conserved amino acid residues in the active site (van der Maarel *et al*., 2002). With these notable features of the alpha-amylases, they are highly significant in many industries nowadays including but not limited to biorefinery, detergent manufacturing, food, medicine, textile, paper industry and pretreatment of animal feed to improve digestibility (van der Maarel *et al*., 2002; Gupta *et al*., 2003; Xian *et al*., 2015). Its increasing demand as one of the key industrial enzymes can also be attributed to the growing environmental problems of using chemical hydrolysis of starch. Previously, starch is being hydrolyzed into glucose using acid, but due to extreme operating conditions detrimental to the environment that needs to be satisfied in the process (such as high acidity and high temperature), this method has been gradually replaced by industrial enzymes such as alpha-amylases to yield high quality fructose syrup from starch (Sundarram *et al*., 2014).

Due to the extensive commercial application of the alpha-amylases in an array of industries, its large-scale production has been explored and developed for the past few decades. Extraction methods have been refined in many ways which basically demonstrated the ubiquity of this enzyme in plants, animals and microorganisms. This enzyme has been successfully isolated from rice (*Oryza sativa* L.), barley (*Hordeum vulgare* L.) and fermented cassava (*Manihot esculenta* Crantz) (Oboh, 2005). In the animal kingdom, alpha amylase has been found to be present in most Bilaterian animals, sponges (*Reniera* sp.), in *Nematostella vectensis*, a sea anemone (Da Lage *et al*., 2007) and even in the carnivorous crustacean spiny lobster (*Panulirus argus*). A Dictyo-type alpha-amylase has also been found from several protists including *Physarum polycephalum*, *Acanthamoeba castellanii Mastigamoeba balamuthi*, *Hartmanella vermiformis* (Da Lage *et al*., 2007). However, microbial alpha-amylases specifically from bacteria and fungi are the most preferred sources for industrial production (Gupta *et al*., 2003; Sundarram *et al*., 2014). This is basically due to their stability as compared to plant and animal alpha-amylases, comparatively cheaper and economical and easy to isolate and extract (Gupta *et al*., 2003; Tanyildizi *et al*., 2004). Moreover, microorganisms are easy and rapid to grow and handle which can be equated to boost in enzyme production.

They can also be genetically engineered for strain improvement, mutations and other changes which are all necessary in obtaining an alpha-amylase with desired characteristics (de Souza *et al*., 2010; Sundarram *et al*., 2014). In the past few years, several bacteria, fungi and other microorganisms have been identified to be capable as a source of alpha-amylases. For the mold species, among that were previously identified were *Aspergillus oryzae* (Sivaramakrishnan *et al*., 2007), *Aspergillus niger* (Djekrif-Dakhmouche *et al*., 2005), *Aspergillus kawachii* (Sudo *et al*., 1994), *Pycnoporus sanguineus* (Almeida *et al.,* 1997) and several *Penicillium* species such as *P. fellutanum* (Kathiresan et al., 2006), *P. expansum P. chrysogenum* (Balkan and Ertan, 2005) and *P. janthinellum* (Sindhu *et al*., 2009) among others. Fungal alpha-amylase has also been isolated from the ascomycetous yeast *Saccharomyces kluyveri* but in a glucose-limited cultivations (Moller *et al*., 2004). For the bacterial species, commercial alpha-amylase has been derived and isolated from several species of bacteria representing the genus *Bacillus*. This includes *B. subtilis* (Konsoula *et al*., 2007), *B. amyloliquefaciens* (Haq *et al*., 2010), *B. coagulans, B. licheniformis*, *B. stearothermophilus*, and *B. cereus* (Anto *et al*., 2006). Due to their high thermostability, alpha amylase produced from *Bacillus* species is the top choice for industrial and large-scale production, and optimization efforts have been employed lately to maximize production (Nielsen and Borchert 2000). Other known source of bacterial alpha-amylase was mainly from the genus *Clostridium* which includes *C. acetobutylicum* (Paquet *et al.,* 1991), *C. thermosulfurogenes* (Swamy and Seenayya, 1996), *C. perfringens* (Shin *et al*., 1995), *C. thermohydrosulfuricum* (Melasniemi, 1995). Moreover, a maltotriose-producing alpha-amylase from the bacteria *Streptococcus bovis* has been characterized and purified by Satoh *et al*., (1997). In general, industrial enzymes such as alpha amylase are being produce through submerged fermentation and solid-state fermentation. The former is the traditional system of producing industrial enzyme and the method of choice for large scale production due to its capability to easily control different parameters such as pH, temperature, aeration and oxygen transfer and moisture (de Souza and Magalhaes, 2010). The latter on the other hand has been recently introduced and emerged as the most suitable method of producing enzymes and other thermolabile products due to its superior productivity performance, employs simple technique, lower capital investment, lower energy requirement, less water output, better product recovery and lack of foam buildup (de Souza and Magalhaes, 2010).

In the present study, we utilized *Bacillus amyloliquefaciens* NBRC strain 15535 to produce alpha amylase. Compared to other species of *Bacillus* such as *B. licheniformis* and *B. subtilis,* it has been reported that *B. amiloquefaciens* produces higher amount of alpha amylase (Roychoudhury *et al.,* 1988), hence the *Bacillus* species used in the experiment. The study also explored the possibility of using taro or cocoyam (*Colocosia esculenta* L.) as an alternative starch source in the Amylase Production Medium, with the general goal of lowering the overall production cost of this enzyme. Taro is a perennial herbaceous plant belonging to the monocotyledonous family Araceae generally grown for food worldwide but is of particular significance in developing countries in Asia, Pacific Island countries, Africa and Central America (Deo *et al*., 2009; Mwamba *et al.,* 2016). This root crop contains a relatively high amount of starch and can be utilized the same way as yam (*Dioscorea alata* L.) and other tubers but generally considered as low value because they are usually consumed unlike other crops such as rice and corns, hence with very little or limited studies to improve its relevance to Agriculture (Falade and Okafor, 2012). Moreover, the effect and influence of different operational conditions and parameter such as pH, temperature, and substrate concentration on the activity of alpha amylase has been studied to determine the most ideal production conditions and parameters. Selection of production conditions were accomplished using statistical tools such as 2-Level Factorial Design with 3 factors of the Stat-Ease Design-Expert software in both laboratory and scale up production. The influence of these parameters on

alpha-amylase activity and biomass production has been investigated and examined to come up with low cost but high yielding method of alpha-amylase production. Most importantly, the present work aims to establish and describe the optimum condition need in alpha amylase production which can be utilized and applied in large scale production to meet the increasing demand of the several industries.

MATERIALS AND METHODS

Microorganism and Culture Media Used

Bacillus amyloliquefasciens NBRC strain 15535, an amylase producing bacterium, was obtained from National Institute of Molecular Biology and Biotechnology (BIOTECH-UPLB). The strain was maintained in Nutrient Agar (NA) slants at 4ºC. The standard medium for inoculum growth was Nutrient Broth (NB). The NB contains tryptone for the nitrogen source, peptone for the carbohydrate and vitamin sources, NaCl for maintaining osmotic equilibrium between the medium and the bacterial cell. Nutrient Agar contains the same ingredients as NB but with agarose gel to solidify the medium. Moreover, Amylase Production Medium was used for fermentation (peptone, MgSO4·7H2O, KCl and Taro starch) (Table 1).

Table 1. Culture Media used in the study.

Culture Media		
Nutrient Broth (1-L)	Peptone	5 g
	Beef Extract	1 g
	Yeast Extract	2 g
	NaCl	5 g
	Distilled water	up to 1L
	*Dehydrated medium: 13 g/L	
	In this study, the dehydrated NB medium was used.	
Nutrient Agar (1L)	Nutrient Broth	13 g/L
	Agar	17 g/L
	Distilled water	up to 1L
Amylose Production Medium (1L)	Peptone	6.0 g
	$MgSO_4 \cdot 7H_2O$	0.5 g
	KCl	0.5 g
	Taro	10 g
	Distilled water	up to 1L

Design of the cell culture bioproduction conditions

For the design of the cell culture bioproduction conditions (pH, temperature and substrate concentration) 2-Level Factorial Design with three factors of Stat-Ease Design-Expert software was used. The values obtained for pH were 4.0, 6.0, and 8.0; for temperature 25°C, 37.5°C, and 50°C and for substrate concentration; 2%, 4% and 6%. The software generated 10 different combinations for every condition with a replicate which resulted in 20 runs These conditions were used in the laboratory-scale fermentation process to determine the optimized conditions for scale-up fermentation. For the scale-up, factorial analysis was used to determine the most optimum condition for fermentation based on the results from the small-scale experiment: pH 8.0, 50ºC and 4% substrate concentration.

Laboratory-scale experiment

A loopful of bacteria from NA slants were incubated to a 50 mL nutrient broth in a 250 ml Erlenmeyer flask kept at 37 ºC for 24 hours. Then, 1 ml of the bacteria was transferred in a 250 ml Erlenmeyer flask containing 50 ml amylase production medium with different pH and substrate concentration. These were incubated in different temperatures (Figure 1) for 24–72 hours. Samples for each run was drawn aseptically to an Effendorf tube for enzyme assay and biomass estimation. The results were used to determine the conditions for the scale-up experiment using 2-Level Factorial Design with 3 factors of the Stat-Ease Design-Expert software.

Scale-up experiment

The inoculum was grown in 250 mL Erlenmeyer flask containing 150 ml Nutrient broth for 24 hours. This was used as the seed stock (5% v/v) for the amylase production. A 3 l bioreactor was used for the scale-up experiment. Sterilization was done in an autoclave at 121ºC, 15 psi for 15 minutes. From the result of the response surface methodology, the conditions for the fermentation process were determined – pH 8.0, 4% Taro as main substrate, and 50ºC. The experiment was performed in two 3 l bioreactors with two different agitation rates in which Bioreactor A at 100 rpm and Bioreactor B at 200 rpm. The aeration rate in both bioreactors was 1 ppm. These conditions were stabilized before inoculation. Samples were drawn every 2 hours for 24 hours in sterile 15 ml Falcon tubes and stored at 4ºC for enzyme assay and biomass determination.

Enzyme Assay

The enzyme activity was tested on the lab-scale and the scale-up experiment. The samples drawn from the fermentation process were transferred into 1.5 ml Eppendorf tubes for centrifugation at 10,000 rpm, 4ºC for 10 minutes. Enzyme solution is found in the supernatant. The enzyme solution (1 mL) is transferred to a test tube with 5 mL 1% soluble starch and 1 mL 0.1M citrate-phosphate buffer at pH 6.5, this serves as the reaction mixture for both enzyme assays. The reaction mixture (1mL) was transferred to another test tube that contains 1 mL of 0.1 N HCl for zero minutes and 30 minutes. The enzyme solution was incubated in a 37 ºC water bath for the 30-minutes incubation period. Afterwards, 0.5mL IKI and 10 mL distilled water were added to the tubes for absorbance reading at 620 nm. Control contains 1 mL HCl, 0.5mL IKI, 0.5 mL distilled water + 1% soluble starch. The α-amylase activity is used for the determination of the amount of the enzyme to hydrolysed starch per minute. The hydrolysed starch (mg) was obtained by subtracting the digest absorbance (AD) from the control absorbance (Ac) dividing it with the control absorbance and multiplying by the initial amount of starch in mg. One unit (U) of alpha amylase is defined as the amount of enzyme that will hydrolyse 10 mg of starch. The alpha amylase test was used in lab-scale and up-scale experiments.

Glucoamylase activity was also tested on the lab-scale to determine the amount of reducing sugar using the DNS method. The reaction mixture was mixed with a vortex then

1 mL was transferred to tubes with mL DNS reagent for zero minutes and 30 minutes. The tubes were cooled down for 1 minute and boiled for 5 minutes. Distilled water of about 5 ml was added to the mixture. The absorbance was measured using a UV spectrophotometer at 540 nm. Control contains 1 mL DNS reagent and 1 mL distilled water. One unit (U) of glucoamylase is the amount of enzyme catalysing 1μmol of glucose per minute.

Estimation of Biomass

In the laboratory-scale, samples from each run were drawn at different times – 24, 48 and 72 hours. The biomass was determined by measuring their absorbance at 600 nm and Miles and Misra cell count method in Nutrient Agar plates. Serial dilution was done for viable cell count from $1x10^{-4}$ to $^{-7}$. Plates were incubated at 37ºC for 24 hours and colony counts were determined as CFU/ml. Growth curve from the flask and 3 l bioreactor were compared. For the scale-up experiment, biomass was estimated using Miles and Misra method with dilutions $1x10^{-4}$ to $^{-7}$.

RESULTS AND DISCUSSION

Growth of *Bacillus* sp. on different growth conditions and taro concentration

The ANOVA results of experiments for studying the effect of three independent fermentation variables (pH, temperature and substrate concentration) are presented along with the F-value responses in Table 2. The model F-value of 8.63 and values of “prob > F” less than 0.05 indicated that the model terms are significant. For α-amylase production, temperature is the only significant model. The “lack of fit F-value” of 0.083 implied that the “lack of fit” is not significant.

Table 2. ANOVA on the significance of the model using three variables.

Source	Sum of Squares	df	Mean Square	F value	p-value Prob>F	
Model	0.97	6	0.16	8.63	0.0009	significant
A-pH	0.029	1	0.029	1.54	0.2389	
B-Temp	0.75	1	0.75	40.18	<0.0001	
C-conc	0.056	1	0.086	4.57	0.0537	
AB	1.323E-004	1	1.323E-004	7.070E-003	0.9344	
AC	0.033	1	0.033	1.79	0.2057	
BC	0.069	1	0.069	3.71	0.0780	
Curvature	0.20	1	0.20	10.58	0.0069	significant
Residual	0.22	12	0.019			
Lack of Fit	1.681E-003	1	1.681E-003	0.083	0.7786	*not significant*
Pure Error	0.22	11	0.020			
Core Total	1.39	19				

Responses of α-amylase activity generated were plotted on the z-axis against temperature and pH variables while keeping the substrate concentration and incubation time at constant level. Therefore, nine response surfaces were obtained by considering all the possible combinations. Figure 1C depicts a three-dimensional diagram and a contour plot of calculated response surface from the interaction between temperature and pH

linear increase in α-amylase production was observed when temperature is increased from 25°C to 50°C. However, no decline in the response variable was observed since the peak of production is still at optimum at the highest temperature.

Bacillus amyloliquefaciens is responsible for much of the world production of alpha-amylase and protease. It is closely related to *B. subtilis* which is one of the best characterized organisms in gram positive bacteria. It is safe, stable and widely used in industrial fermentation process (Schallmey *et al*., 2004). Taro (*Colocasia esculenta* L.) is a tropical plant grown primarily for its edible corms (Lim, 2015). It is considered as a tuber that is very rich in carbohydrates, ranging between 73 to 80% which is mainly starch at 77.9% and 1.4% crude fiber, on a Dry Matter (DM) basis. Because of its inherent high carbohydrate composition, this tuber is a major source of diet and represents one of the main sources of energy in many parts of the tropics and sub-tropics providing about a third of the food intake (Soudy *et al*., 2010). However, studies on use of this tuber as carbohydrate source in bioproduction of important industrial enzymes, if none, has been limited.

Stat-Ease Design-Expert software used in the experimental set-up suggested the importance of various fermentation parameters at different levels (pH, temperature and substrate concentration). The methodology employed will be successful to any process, where an analysis of the effects and interaction of many experimental factors are required (Kunamneni and Singh, 2005). This powerful statistical tool could reduce the conventional time-consuming

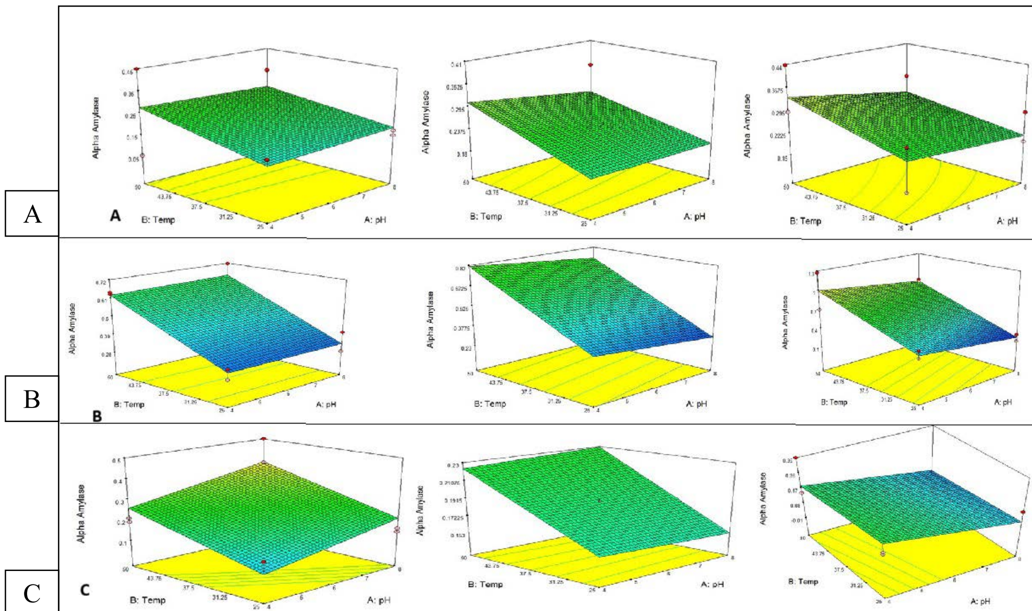


Figure 1. Three-dimensional diagram of the interaction of varying temperature and pH at zero level substrate concentration and incubation time.

and laborious optimization experimental setup. This particular experiment, though not as perfect, could support the claim. In this study, substrate concentration of 4% taro medium, initial medium pH of 8.0 and temperature at 50°C were the major factors that influenced the enzyme titer. The results also show that temperature is the most important drive for the optimum production of enzymes. Temperature is a vital environmental factor which controls the growth and production of enzymes or metabolites that differs from one species of microorganisms to another (Banargee and Bhattacharya, 1992; Kumar and Takagi, 1999). The fermentation variables and substrate used are also similar to the study of Swain and Ray (2007). This baseline data was used for the upscale production of alpha amylase in bioreactor which will be discussed in the succeeding sections.

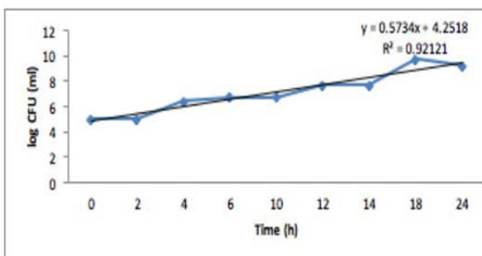
Time-course *Bacillus amyloliquefaciens* growth in taro medium

The time course of *Bacillus* sp. growth on 4% (w/v) taro was investigated as a function of different agitation rates in 3L bioreactor (100 and 200 rpm) using the fermentation parameters as previously discussed. The cell biomass (log CFU/mL) of *Bacillus* sp. plotted against time (h) in both bioreactors (Figure 2). Bioreactor A (100 rpm) shows a linear increase of colony forming unit (CFU) as time progresses. This, on the other hand, is not observed in Bioreactor B (200 rpm). A lag phase was also not detected for both experimental set-ups, probably due to the sampling period used. Furthermore, bacterial culture s did not reach the stationary phase since the system is currently undergoing an exponential phase within 24 hours. Probably, a stationary phase can be detected if the time for fermentation reaction is increased to 48 to 72 hours. Maximum biomass concentration was higher in Bioreactor A (5x109 CFU/mL) than in Bioreactor B (5x108 CFU/mL), suggesting that higher agitation rate limits the number of cells that can be obtained. This may imply that concentration may require specific amount of agitation rate. A high R2 value was observed by fitting a line in the growth curve of the bacterial culture in Bioreactor A. The maximum specific growth rate (umax) was determined to be 0.5745 hr-1 using the equation of the line. This cell kinetics parameter, however, cannot be determined in Bioreactor B as data did not follow a typical cell growth curve. In Bioreactor B, these results show that taro is suitable for *Bacillus amyloliquefaciens* growth in a time scale of more than 24 hours.

Alpha-amylase production by *Bacillus amyloliquefaciens* grown in taro medium

From the time course study in bioreactor, it was found that the rate of enzyme production was increased with the increase of the fermentation period and reached its maximum activity after 12-hour bioproduction in Bioreactor A which means no more changes in the enzyme in the enzyme production after 12 hours. Same trend was also observed in the rate of enzyme

Bioreactor A



Bioreactor B

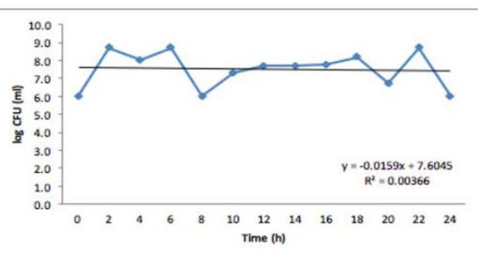


Figure 2. Growth curve of *B. amyloliquefaciens* grown in Bioreactor A and B with varying agitation rate.

production in Bioreactor B, in which the maximum enzyme activity is observed after 14 hours (Table 3).

Table 3. Summary table showing the bioproduction of alpha amylase (U) at time.

Hours	Bioreactor A (U)	Bioreactor B (U)
2	0.138	0.169
4	0.152	0.146
6	0.151	0.18
10	0.112	0.151
12	0.2	0.133
14	0.138	0.192
16	0.173	0.167
18	0.128	0.154
20	0.143	0.154
22	0.083	0.057
24	0.151	0.069

The prolonged incubation time was beyond 12 hours (Bioreactor A) and 14 hours (Bioreactor B) did not increase the enzyme production, rather decreased (Figure 3). This trend is similar to the results reported by Haq *et al*. (2010), Asgher *et al*. (2007), and Kaur and Vyas (2012). Such a result might be caused by the accumulation of other by-products in the medium associated with the growth of bacteria. Efficient enzyme production might not occur until reaching the stationary phase (Huang *et al*. 2003; Wanderley *et al*. 2004). In contrast, study conducted by Abate *et al*. (1999) demonstrated that the production of α-amylase by *Bacillus amyloliquefaciens* starts at the beginning of the exponential growth phase reaching the maximum level after 24 hours, thereafter, α-amylase level decreased drastically probably due to the accumulation of high level of protease activities associated with the bacterial spore formation towards the end of the exponential growth phase. These results suggest that the amylase production is not yet attained with the given 24 hours of incubation of bacterial culture. Since the data on cell kinetics shows that the culture is still undergoing exponential growth, alpha amylase production is still not optimum. Therefore, increasing the incubation time is recommended for increased alpha amylase production.

CONCLUSION

Bacillus amyloliquefaciens is a potential microorganism for the production of amylase. However, there are other factors and parameters to consider in the production of amylase. Result showed that there are differences in the cell number of two bioreactors due to different agitation speed. This only implies that agitation and aeration may affect the cell concentration.

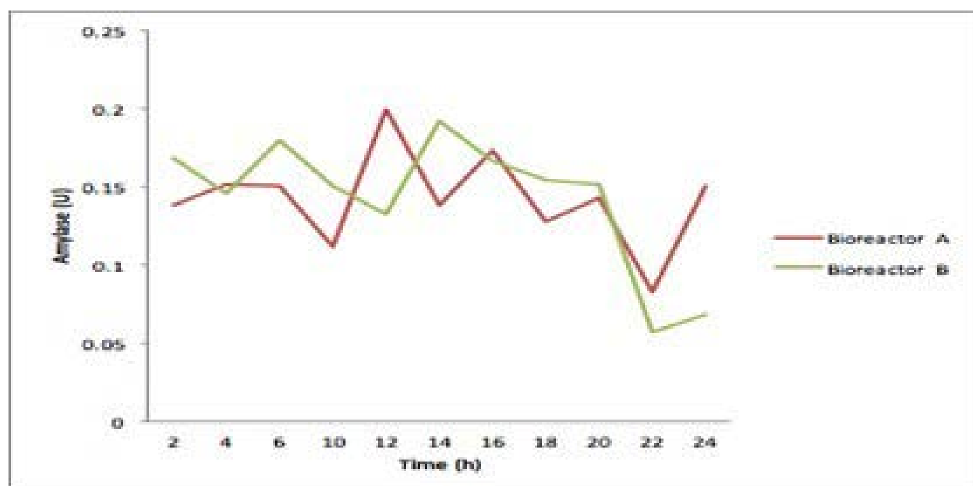


Figure 3. Plot of specific activity of alpha-amylase as a function of time (h).

This study suggests extending the fermentation of incubation time to 48 to 72 hours in order to observe the growth of microorganism and amylase production since the result showed that there is still in the stationary phase.

In addition, pH can also be lowered to four to six since the *Bacillus amyloliquefaciens* prefer quite acidic environments. It would also be better to gather data on substrate and cell concentration through time. Other methods in determination of cell concentration may also be explored.

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