Journal of Advances in Biology & Biotechnology

14(2): 1-18, 2017; Article no.JABB.33698
ISSN: 2394-1081

Kinetic Study of Cell Growth and Production of Amylase, Cellulase and Xylanase by *Bacillus subtilis* Using Barley Husk as the Prime Carbon Source

Hooi Ling Ho[1*] and Xin Ning Ku[1]

[1]Faculty of Applied Sciences, UCSI University, No. 1, Jalan Menara Gading, UCSI Heights, Cheras, 56000 Kuala Lumpur, Malaysia.

Authors' contributions

This work was carried out in collaboration between both authors. Author HLH designed and supervised the research, wrote and edited the manuscript. Author XNK performed the experiments and analysis. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JABB/2017/33698
Editor(s):
(1) Afroz Alam, Department of Bioscience and Biotechnology, Banasthali University, Rajasthan, India.
Reviewers:
(1) Amit Kumar, Maharishi Markandeshwar University, India.
(2) Marcelo Teixeira Leite, Federal University of Paraíba, Brazil.
(3) Mohamed Fadel, National Research Center, Egypt.
(4) Slimane Choubane, University of sciences and technology of Oran, Algeria.
Complete Peer review History: http://www.sciencedomain.org/review-history/20237

Original Research Article

Received 26th April 2017
Accepted 5th July 2017
Published 27th July 2017

ABSTRACT

Bacillus spp have been widely adopted as one of the vital producers of the industrial enzymes including amylase, cellulase and xylanase. In fact, submerged fermentation (SmF) with the presence of excess water is the best suited technique for the culture of bacteria especially *Bacillus* that required high moisture content to grow. Pure carbon sources besides being expensive, are not economically viable for the production of enzymes. Therefore, inexpensive yet effective agricultural residues such as barley husk was used in this study.

Aims: The primary aim of this study was to investigate the kinetic of cell growth and enzymes production of amylase, cellulase and xylanase by *Bacillus subtilis* ATCC 6633 using barley husk as the main carbon source under SmF.

Methodology: In the present study, the standard inoculum size of 1×10^8 cells of *B. subtilis* was inoculated into culture flask containing barley husk for the production of enzymes in SmF. Samples were collected every 12 h for analysis.

**Corresponding author: E-mail: hohooiling@gmail.com;*

Results: In this study, *B. subtilis* possessed the maximum specific growth rate (μ_{max}) of 0.55 h^{-1} at 48 h with the maximum cell productivity of 1.98×10^{10} cells/L/h was attained during the exponential growth phase. On the other hand, the highest enzyme activity by *B. subtilis* obtained in this study was identified to be amylase with its activity of 1.991 U/mL, followed by xylanase activity of 1.492 U/mL and lastly cellulase with the lowest activity of 0.304 U/mL. In addition, the specific enzymes activity and productivity were also elucidated to describe the kinetic study of enzymes production. The maximum specific xylanase activity of 6.81 U/mg, followed by specific amylase activity of 6.68 U/mg and the least specific cellulase activity of 0.73 U/mg were attained from *B. subtilis*. In fact, cellulase productivity of 18.23 U/mL/h was found to be relatively low compared to amylase with 119.48 U/mL/h and xylanase with 89.52 U/mL/h. Cellulase production was determined as growth associated process where its maximum production was attained at the end of the exponential growth phase. On the contrary, the production of amylase and xylanase were partially growth associated due to their maximal production observed after the exponential phase of cell growth.

Conclusion: In a nutshell, *B. subtilis* is anticipated to be potential bacteria for the optimisation of enzymes production for amylase, cellulase and xylanase using barley husk as the sole carbon source in SmF.

Keywords: Amylase; Bacillus subtilis; cell growth; cellulose; submerged fermentation (SmF); xylanase.

1. INTRODUCTION

Microorganisms including bacteria, fungi and yeast are rich sources of enzymes, in fact, microbial enzymes are more preferable over plants and animals due to their economic feasibility, high yields and consistency, ease of product modification and optimization, regular supply due to absence of seasonal fluctuations, rapid growth of microbes on inexpensive media, stability, ease cultivation in large fermenters and greater catalytic activity. Majority of enzymes currently used in industry are of microbial origin which has shown tremendous potential for different applications such as amylase, cellulase and xylanase. Amylase is an enzyme involves in the hydrolysis of starch results in the production of dextrin, maltose and finally glucose. Production of amylase is usually performed by *Bacillus subtilis.* Besides that, xylanase is induced by xylan to break down xylose. *Bacillus* sp. was a good producer of xylanase. Xylanase from microorganisms have attracted attention for the last few decades due to their biotechnological potential in various industrial processes including food, baking, animal feed, pulp and paper industry. On the other hand, *Bacillus sp* also produces cellulases. Cellulase produced by microorganism is a synergistic enzyme that is used to break down cellulose to glucose or other oligosaccharide compounds. In recent years, cellulase possesses wide applications in variety of fields such as textile, paper and pulp, food and animal feed, pharmaceutical, fuel and chemicals industry. In conclusion, *Bacillus* spp are one of the interesting industrial microorganisms due to their rapid growth rates and ease in manipulation that leading to a short fermentation cycle besides their capacity to produce important enzymes such as amylase, cellulase and xylanase. Submerged fermentation (SmF) is a process that utilizes free flowing liquid substrates for growth of microorganisms especially *Bacillus* sp. SmF is one of the best suited fermentation modes for *B. subtilis* which requires relatively high moisture content. SmF is preferable due to its high efficiency of purification of products and also more efficient and effective in transfer of heat and mass. Furthermore, suitable growth conditions and sufficient nutrients in SmF allow proper proliferation of microorganisms to produce high yield of enzymes. In fact, carbon source is the most important prerequisite nutrient for growth of microorganisms in SmF. Nonetheless, pure substrates containing carbon sources which being highly expensive could not be affordable for the production of enzymes. Consequently, to lower production cost and to enhance enzymes production, inexpensive yet effective carbon sources such as agricultural residuals are commonly introduced and applied. Such agricultural residuals included various raw materials such as sawdust, poplar trees, sugarcane bagasse, waste paper, brewer's spent grains, switch grass, straws, stems, stalks, leaves, husks, shells and peels from cereals, rice, wheat, corn, sorghum and barley. Barley husk is a cereal waste which is commonly used due to its advantage of light weight, specific modulus,

non-toxic, high sugar content, ease in processing and renewable, hence it is suitable for fermentation as prime nutrient [1,2]. Enzyme synthesis and production is a complex process, where the final quality and quantity of the product yield depends on microorganism strains, metabolic pathways, fermentation parameters, enzyme production phases, carbon and nitrogen sources and nutrient depletion conditions. Thus, kinetic model in SmF is substantially capable to detect cell growth and enzymes production. The mathematical kinetic models facilitate data analysis and provide strategy for solving problems encountered in enzymes fermentation. Therefore, in this study, SmF was used to estimate kinetic parameters for cell growth and enzymes production by *B. subtilis*. In this respect, the information on kinetics model is potentially important for the improvement of fermentation enzymes performance. Based on the previous study by Ho [2], this study elucidated the kinetics models of cell growth and production of enzymes including amylase, cellulase and xylanase by *B. subtilis* under SmF using barley husk as the prime carbon source.

2. METHODOLOGY

2.1 Nutrient Agar

Nutrient agar was prepared and autoclaved at 121℃ for 15 min before it was poured on the sterile Petri dishes. A control agar plate was incubated at 37℃ for two days to observe for any microbial contamination. Then, the nutrient agar plates were stored in the chiller at 4℃ to be used for submerged fermentation.

2.2 *Bacillus subtilis* ATCC 6633

Bacillus subtilis ATCC 6633 was obtained from American Type Culture Collection (ATCC), Manassas, USA. Subculture of bacteria was left cold before it was used to pick up a single colony of the mother culture and streaked on the nutrient agar. After that, the streaked pure culture plates were sealed with parafilm prior incubated at 37℃ until the formation of colonies appeared after two days of incubation. Extra culture plates were stored in the chiller at 4℃ and remaining culture was subcultured into nutrient broth to ensure the continuous growth of the culture at regular time intervals. A sufficient amount of bacterial culture from nutrient agar was aseptically transferred into the nutrient broth and incubated at 37℃ for two days before it was ready to use.

2.3 Pre-treatment of Barley Husks

Barley husk was utilized as the prime carbon source for the growth of *B. subtilis* in SmF. Barley husk was oven-dried at 65℃ until constant weight was achieved. Then, the dried barley husks was grounded with a blender followed by sieving to produce consistency in size prior subjected to sterilization by autoclaving at 121℃ for 15 min. This process was carried out to ensure *B. subtilis* possessed better accessibility to the carbon source of the barley husk. The processed barley husk was then kept in dry condition for further use.

2.4 Preparation of Standard Inoculum and Optimum Medium and Growth Conditions

The medium composition used in the study was composed of (g/L): yeast extract, 5; peptone, 5; $MgSO_4 \cdot 7H_2O$, 0.1; K_2HPO_4, 1 and pre-treated barley husk, 20 which employed as the prime carbon source for the production of enzymes [2]. Barley husk was used as the main carbon source while other medium compositions possessed as nitrogen source, vitamins and trace elements of minerals for the optimum growth and enzymes production from *B. subtilis.* 250 mL was used as the standard working volume of medium in a sterile 500 mL Erlenmeyer flask. Total of four culture flasks were used in quadruplicate. Barley husk was prepared separately from nitrogen source and other medium compositions to prevent Maillard reaction. Then, the initial medium pH of both carbon and nitrogen sources was adjusted to 6.5. The carbon source was autoclaved separately from other compositions. Both of the carbon and nitrogen sources with other medium compositions were mixed together in the Erlenmeyer flask. Bacteria culture from the nutrient broth was used for the preparation of cell suspension. A cell suspension of 1×10^8 was required as the standard inoculum size for the growth and production of enzymes throughout the whole study. Hence, a serial dilution of cell suspension was carried out in order to obtain a final cell concentration of 1×10^8 cells. The number of bacteria cells was counted using haemocytometer under a microscope. After the medium was inoculated with 1×10^8 cells, the mouth of the Erlenmeyer flask was then plugged with the non-absorbent dried cotton wool After that, the bacteria culture was incubated in an orbital rotary shaker operating at 37℃ agitated at 150 rpm. Sampling was conducted at the

regular time interval of 12 h from the culture flasks for analysis.

2.5 Sampling and Data Analysis

Samples of *B. subtilis* were harvested and collected at every 12 h interval over a period of 120 h. The samples were collected for the quantification of biomass concentration and measurement of medium pH prior subjected to centrifugation at 10,000 rpm for 15 min to obtain clear supernatant. The enzymes activity by *B. subtilis* including amylase, cellulase and xylanase were performed and analysed using 3,5-dinitrosalicyclic acid (DNS) method [3]. All of the experiments were carried out independently in quadruplicate and the results presented are the mean of four results that were generated from the experiments with standard deviation (SD) calculated. Hereafter, analysis of the enzymes production and kinetic parameters were conducted. The fermentation data were then evaluated with the proposed models to generate kinetic parameters values. All enzymes productivity attained were compared using proposed equations to estimate the values of maximum specific growth rate (μ_{max}), maximum biomass concentration (X_{max}), maximum cell productivity, doubling time (Td), maximum enzyme production, specific enzyme activity and maximum enzyme productivity for amylase, cellulase and xylanase.

2.6 Amylase Activity Assay

Amylase activity was determined by method according to Bhatnagar et al. [4] was adopted herein for the amylase activity assay where 1% (w/v) starch was used as the substrate. Amylase activity was determined by adding 1 mL of enzyme solution to 2 mL of 1% starch solution and 2 mL of 0.2 M sodium acetate buffer, pH 4.8. Then, the reaction mixture was incubated at 37℃ for 10 min. 1.5 mL DNS was added into the mixture and incubated at 90℃ for 5 min. The amount of liberated reducing sugar was measured using UV-VIS spectrophotometer at 575 nm. One unit of amylase activity was defined as the amount of enzyme required to release one µmole of maltose per ml of enzyme extract per min under assay condition.

2.7 Cellulase Activity Assay

In this study, the cellulase activity was determined by measuring the glucose released during carboxymethyl cellulose (CMC) hydrolysis. A modification of reducing sugar method according to Chinedu et al. [5] was adopted herein for the cellulase activity assay. 0.1% (w/v) CMC was used as the substrate of the cellulase activity assay. To perform the cellulase activity, 2 mL 0.1% (w/v) CMC in 0.1 M sodium acetate buffer, pH 5.0 was added to 2 mL enzyme solution. Then, the mixture was incubated at 37℃ in water bath for 30 min. 1.5 mL DNS was added into the mixture and incubated at 90℃ for 5 min. The absorbance was measured at 575 nm against an enzyme free control. The amount of liberated reducing sugar was determined by DNS where one unit of cellulase activity was defined as the amount of enzyme required to release one µmole of glucose per ml of enzyme extract per min under assay condition.

2.8 Xylanase Activity Assay

Xylanase activity was determined by measuring the xylose released during xylan hydrolysis. A modification of reducing sugar method according to Bailey et al. [6] was adopted herein for the xylanase activity assay. 1% (w/v) beechwood xylan was used as the substrate in this assay. 0.1 mL supernatant was added with 0.9 mL 1% (w/v) xylan in 0.05 M sodium phosphate buffer, pH 5.3 and incubated at 50℃ in water bath for 30 min. 1.5 mL DNS was added into the mixture and incubated at 90℃ for 5 min. The absorbance was measured at 575 nm against an enzyme free control. To quantify the xylanase activity, one unit of xylanase activity was defined as the amount of enzyme required to release one µmole of xylose per ml of enzyme extract per min under assay condition.

2.9 Protein Assay

Determination of the total amount of soluble protein in the supernatant was measured at 750 nm according to Lowry et al. [7].

2.10 Quantification of Biomass Concentration

Cell count of *B. subtilis* was estimated using a haemocytometer. Cell suspension from the sample was withdrawn and serially diluted before transferred to a sterile haemocytometer for cell count. 20 µL well mixed aliquot sample was transferred to the haemocytometer with a glass coverslip on top of the grid using autoclaved pipette tips. Many of the rod shape *B. subtilis* were observed and counted under phase contrast microscope using haemocytometer.

2.11 Measurement pH of Culture Medium

The pH of culture medium was measured using an electronic pH meter after calibrated with buffers of pH 4, 7, and 10.

3. RESULTS AND DISCUSSION

3.1 Cell Growth and Enzymes Production by *B. subtilis* Using Barley Husk as the Prime Carbon Source in Submerged Fermentation (SmF)

The optimum production of amylase, cellulase and xylanase by *B. subtilis* using barley husk as the primary substrate under optimum growth conditions of initial medium pH at 6.5 at 37℃ with the agitation of 150 rpm [2]. Under these growth conditions, proper mixing, mass and heat transfer were satisfied. Data presented shows that *B. subtilis* produced amylase, cellulase and xylanase in the optimal conditions under SmF. Numerous researchers utilized SmF for microbial enzymes production in the presence of excess liquid medium which is best suited for *B. subtilis* that required high moisture content. Besides that, a standard optimum temperature is crucial to regulate the enzymes production during the fermentation process. Although Thangam and Rajkumar [8] reported that *Bacillus* species possessed high temperature stability, nonetheless, temperatures which are higher than the optimum range would denature the cells whereas those lower than the optimum range would otherwise inactivate the cells, hence influence the production of enzymes. The standard optimal growth temperature of 37℃ with optimum agitation speed of 150 rpm for *B. subtilis* was also used in the study conducted by Sanghi et al. [9] and Bozic et al. [10] which yielded 298 U/mL of xylanase and 1.55 U/mL of amylase, respectively. On the other hand, Budde et al. [11] stated that *B. subtilis* was also able to sustain cell growth in the temperature range from 11℃ to 52 ℃ under laboratory conditions. Besides that, enzymes production is also greatly depended on the initial pH of the medium. In this study, *B. subtilis* was apparently able to thrive in a slightly acidic environment of pH 6.5 to flourish during the start of fermentation process where the pH 6.5 was a tolerable pH range that suitable to initiate the secretion of enzymes. The standard optimum initial medium pH of 6.5 for *B. subtilis* was applied by Ho [12]. It was also agreed by Abdelwahed et al. [13]. The sole carbon source used in the SmF for the enzymes production was barley husk as the prime nutrient for *B. subtilis.* Barley husk is mainly composed of lignocellulosic cell wall with huge composition of polysaccharides consisted of 39% cellulose, 22% lignin, 12% hemicelluloses, 11% starch and small amount of fibers, proteins, resins and antioxidants [14]. Due to the lignocellulose contents in barley husk, amylase was secreted by *B. subtilis* to hydrolyse starch. Cellulase was also being produced to hydrolyse carboxymethylcellulose in barley husk. Additionally, xylan as part of hemicelloloses found in barley husk was degraded by xylanase. Hence, barley husk that contains bound sugars has been promoted and designated as the fermentation raw material for the maximum production of enzymes. Sampling analysis was carried out to elucidate the growth and maximum production of enzymes from *B. subtilis* under SmF.

3.2 Growth Profile of *B. subtilis*

In the present study, the biomass concentration of *B. subtilis* was measured using haemocytometer to illustrate the growth profile of *B. subtilis* during enzymes production in SmF. Fig. 1 illustrates the cell growth profile during the production of enzymes via SmF. In this study, *B. subtilis* shows a very minimal growth during the lag phase which occurred between 0 h to 12 h of SmF. In case of growth conditions, the short lag phase is an adjustment period during which bacterial cells adapted themselves to encounter the changes in the newly growth environment, including pH of medium, temperature and agitation speed [15]. Thereafter, cell density shows a steeper increase in the gradient from $6.48 \times 10^7 \pm 7.89 \times 10^6$ cells/mL at 12 h to $9.53 \times 10^8 \pm 4.99 \times 10^7$ cells/mL at 48 h as shown in Fig. 1. The enzymes synthesis and secretion started during the rapid logarithm phase (exponential phase) of *B. subtilis* which took place between 12 h to 48 h. Based on the result, the bacterial culture possessed the maximum specific growth rate (μ_{max}) of 0.55 h^{-1} at the logarithm phase with the generation time (Td) of approximately 76.80 min. During this time, the bacteria was apparently hitting its stride, hence doubling its number every 76.80 min. Certain enzymes production including cellulase increases with the specific growth rate, approaching its maximum activity at the highest growth rates. Consequently, the maximum biomass concentration (X_{max}) in the experimental data was $9.53 \times 10^8 \pm 4.99 \times 10^7$ cells/mL at 48 h with the maximum cell productivity of 1.98×10^{10} cells/L/h. After the

depletion of critical growth substances from the liquid medium, the increase in cell mass eventually ceased before entered to the death phase. Apparently, the biomass production of *B. subtilis* entered to the death phase rapidly which produced $8.05 \times 10^8 \pm 4.43 \times 10^7$ cells/mL at 60 h. Bansal et al. [16] mentioned further fermentation process possessed decline in the biomass and enzymes yield, which probably a consequence of random lethal events, involving cellular fragmentation in the death phase and release of intracellular toxic materials into the fermentation broth. According to Fig. 1, the bacterial growth declined significantly from 60 h to 84 h to reach to the minimum. Nonetheless, constant decreased of cell number after 84 h to 120 h indicating the end of fermentation process where loss of cell mass occurred as a result of cell death.

Extracellular protein production was conducted in the conical flasks using Lowry assay to analyse the quantitative measurement of total soluble protein in the samples. It was highly desirable and simpler, yet easily replicable methods to assess protein concentration. In Fig. 2, it shows the soluble protein production by *B. subtilis* during SmF where the protein production was closely related to the cell growth. The bacterial culture entered the logarithm phase with an increase in cell protein from 0.370 ± 0.006 mg/mL at 12 h to 0.417 ± 0.007 mg/mL at 48 h. There was approximately an increment of 12.7% protein concentration during the logarithm phase. In this study, the highest production of the protein was 0.417 ± 0.007 mg/mL produced by $9.53 \times 10^8 \pm 4.99 \times 10^7$ cells/mL at 48 h of SmF. As the fermentation time prolonged, the protein concentration decreased enormously to 0.270 ± 0.006 mg/mL at 72 h and remained approximately at 0.216 mg/mL from 84 h to 108 h followed by a decline to 0.195 ± 0.006 mg/mL at 120 h. Based on this study, apparent changes in culture pH of medium also occurred during the growth of *B. subtilis* using barley husk as the prime carbon source under SmF. pH increased from an initial value of 5.43 at 12 h to 9.13 at the end of the fermentation period as depicted in Fig. 3. During the logarithm phase, $6.48 \times 10^7 \pm 7.89 \times 10^6$ cells/mL was produced at pH 5.43 at 12 h, thereafter, $9.53 \times 10^8 \pm 4.99 \times 10^7$ cells/mL at pH 7.48 at 48 h of SmF. Correspondingly, Logan and de Vos [17] reported that *B. subtilis* was able to thrive at pH ranging from 5.5 to 8.5. As the medium pH continuously increased after 48 h, the prolonged fermentation resulted

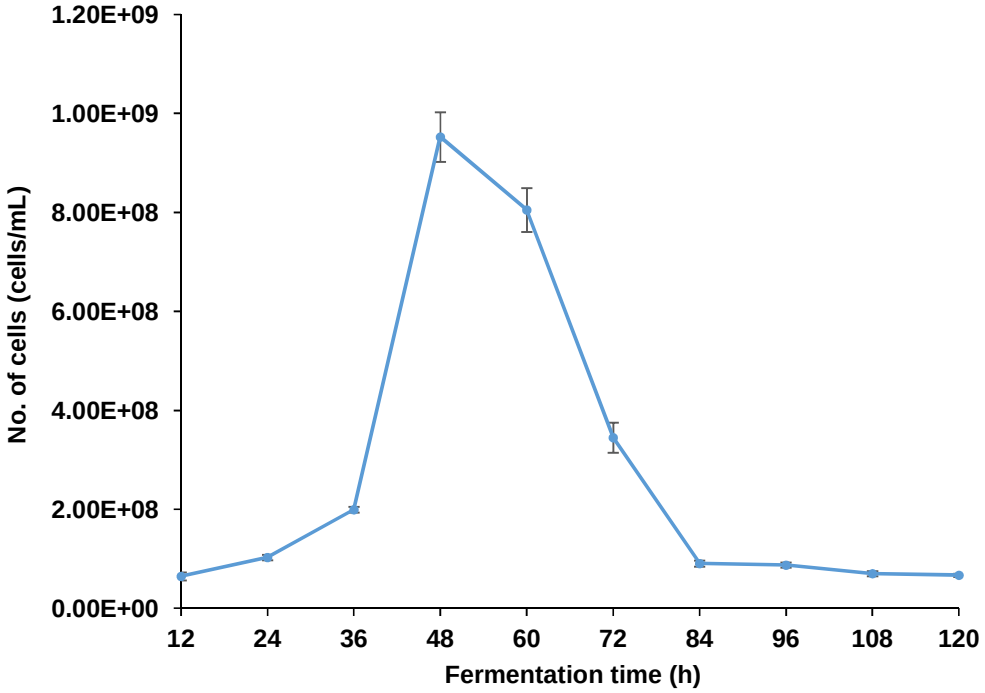

Fig. 1. Growth profile of *B. subtilis* during production of enzymes using barley husk as the carbon source in SmF

in getting poorer bacterial growth. According to Vijayalakshmi et al. [18], increase or decrease of medium pH in either side of the microbial culture would result in poor microbial growth. Variations in pH of medium occurs result from protein hydrolysis and metabolite production. Proteases secreted by *Bacillus* spp decompose proteins which lead to the release of ammonium, a compound that increases medium pH. Apparently, when the medium environment becomes more alkaline, it results base stress to the bacterial cells and hence, the growth is terminated and endospores are formed generally [19]. Thus, it has been suggested that the metabolic activity of bacteria is very sensitive to the pH level of medium.

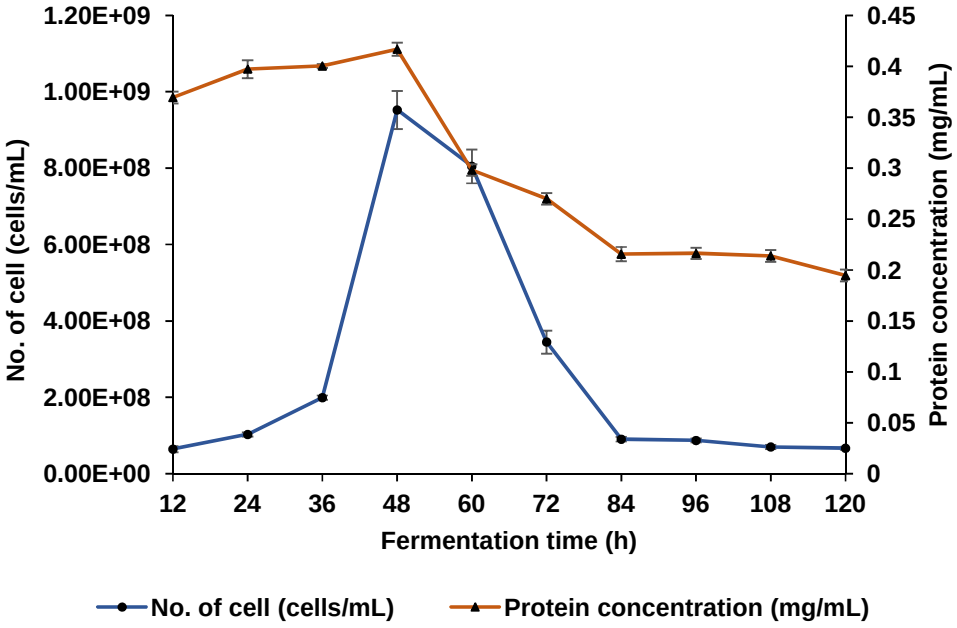

Fig. 2. Correlation of cell growth with the protein production by *B. subtilis* over fermentation time in SmF

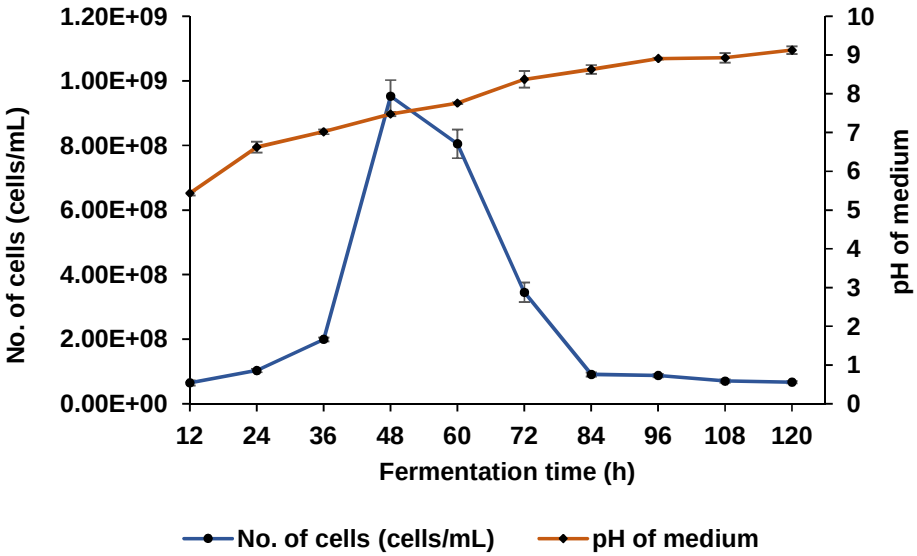

Fig. 3. Correlation of cell growth of *B. subtilis* with the pH of medium over fermentation time in SmF

3.3 Amylase Production by *B. subtilis*

The production of amylase by *B. subtilis* in SmF under optimum growth conditins in this study is shown in Fig. 4. Biomass production was greatly influenced the production of amylase as shown in the correlation graph between the production of biomass and amylase activity in Fig. 4. In this study, the positive relationship between the cell growth was found to be proportional to the amylase production. Amylase activity increased by 2.923 fold from 0.610 ± 0.046 U/mL at 12 h to 1.783 ± 0.042 U/mL, producing $2.00 \times 10^8 \pm 5.80 \times 10^6$ cells/mL at 36 h. Thereafter, amylase production was increased to 1.831 U/mL produced by $9.53 \times 10^8 \pm 4.99 \times 10^7$ cells/mL at 48 h in SmF. The microbial amylase reached its maximum activity of 1.991 ± 0.084 U/mL produced by $8.05 \times 10^8 \pm 4.43 \times 10^7$ cells/mL with its maximum productivity of 119.48 U/mL/h at 60 h of SmF. Apparently, the optimum time of enzyme synthesis was detected at 60 h of fermentation. Incubation beyond the optimum time course was generally accompanied by a decrease in the amylase activity and growth rate, which gradually declined to 1.202 ± 0.075 U/mL by $6.70 \times 10^7 \pm 3.56 \times 10^6$ cells/mL after 120 h of fermentation. Consequently, based on this study, it was demonstrated that the prolonged fermentation time possessed an adverse effect on the progress of the bacterial growth in submerged state. Indeed, the decrease in amylase activity after 60 h of SmF might due to the denaturation and decomposition of amylase as a result of interaction with other components such as proteinase present in the liquid medium as reported by Ozdemir et al. [20]. Similarly, according to Bozic et al. [10], the maximum production of amylase of 1.55 U/mL at 8 h using soluble starch as the carbon source was found to decline gradually because of the simultaneous production of peptidases which deceased the amylase activity during the fermentation time. Nonetheless, the production of amylase that is secreted by *Bacillus* spp primarily occur at the end of trophophase or during the relatively short stationary growth phase where their rates of activity are not directly related to the growth rate of the cells. Therefore, the growth of the cells might have reached a stage that indirectly stimulates the production of secondary metabolites [21]. On the other hand, optimum amylase production and activity that were reported by several researchers occurred at different incubation period of fermentation. The production of amylase in SmF reached its maximum of 4 U/mL at 10 h of incubation period was reported by Vidyalakshmi et al. [22]. Further increase in incubation period did not show any significant increase in amylase production rather it showed otherwise. According to Akcan et al. [23], their results at 72 h of SmF was the optimum incubation period for the maximumα-amylase production which yielded

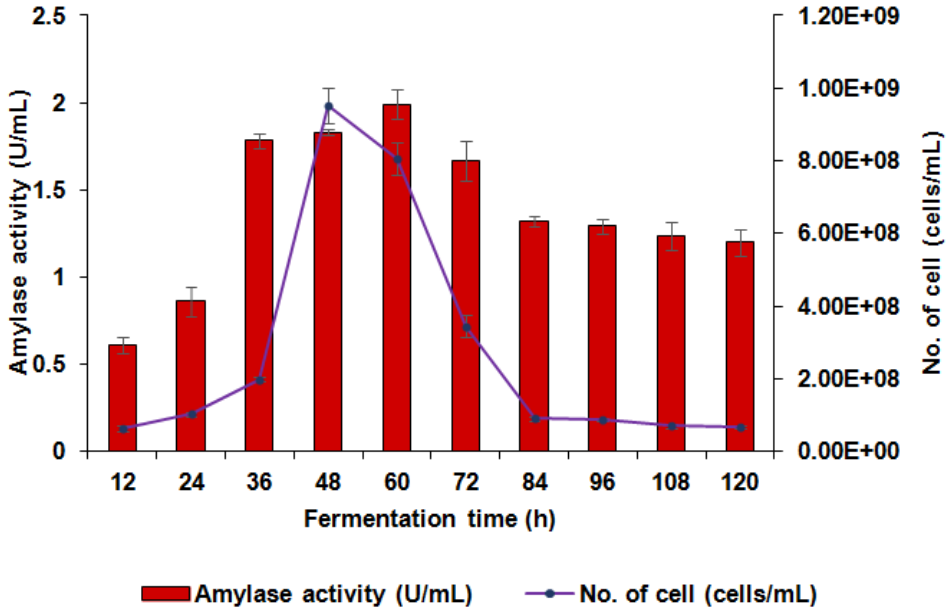

Fig. 4. Correlation of amylase activity with the number of cells over fermentation time by *B. subtilis* in SmF

858.6 ± 41.9 U/mg. Nevertheless, the enzyme production gradually declined after 72 h of SmF in this study. Therefore, depending on different *Bacillus* strains and environmental conditions, amylase could become constitutive or inducible enzyme, showing different incubation period for its maximum activity pattern.

Based on Fig. 5, it shows the relationship between soluble protein production by *B. subtilis* and amylase activity over fermentation time under SmF. Protein concentration showed constant increase within the 48 h as well as the production of amylase that increased gradually within the first 60 h. As the fermentation time prolonged, it showed amylase activity gradually decreased with the protein concentration from 72 h to 120 h of fermentation due to the depletion of nutrients in the medium. Even though, the enzyme that was elucidated in this study was a crude enzyme, nevertheless, assay on protein concentration was included in this study to estimate the specific enzyme activity of amylase, cellulase and xylanase, respectively. Specific enzyme activity is a term used for the estimation of enzyme purity. The value of specific enzyme activity becomes larger when the enzyme preparation becomes purer with lesser of the amount of protein. Indeed, the larger the amount of specific enzyme activity, the purer the enzyme. Based on the result obtained in this study, the highest activity of amylase produced in SmF was found to be 1.991 ± 0.085 U/mL with its specific activity of 6.68 U/mg of protein at 60 h of fermentation. The amylase produced in the present study was relatively pure, nonetheless, futher purification procedures were needed. Hence, there was interconnected relationship between protein concentration with its specific enzyme activity.

Most microorganisms multiply optimally within a wide pH range. From Fig. 6, the culture medium pH generally shows constant increase until the end of fermentation where the culture medium became more alkaline. pH of the medium increased from 5.43 ± 0.05 to 7.76 ± 0.02 which triggered amylase production from 0.610 ± 0.046 U/mL at 12 h to 1.991 ± 0.085 U/mL at 60 h. Hence, this result suggests that the maximum amylase activity occurred at slight alkaline condition of pH 7.76 at 60 h under SmF using barley husk as the substrate. Similarly, Riaz et al. [24] reported that the maximum yield of amylase by *B. subtilis* occurred at pH 7.5 using pearl millet starch as the carbon source. Furthermore, amylase was also stable from pH 6.5 to pH 8.0 at the incubation temperature of 40℃. Another study from Riaz et al. [24] reported that the medium pH between 7.5 and 8.0 were the optimum pH for the production of amylase by *B. subtilis*. Based on the study, a further increase

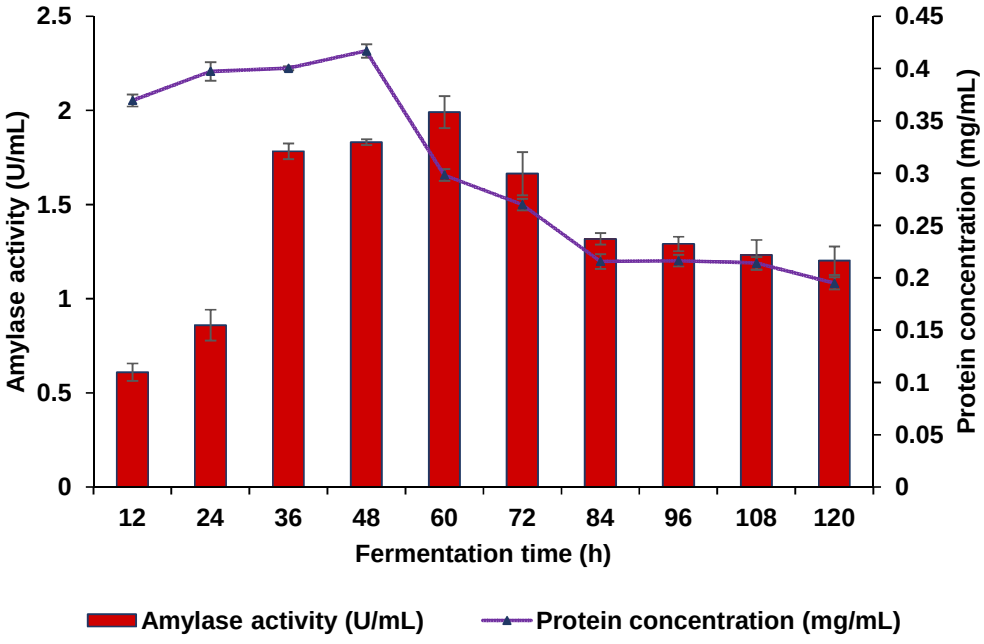

Fig. 5. Correlation of amylase activity with the protein concentration over fermentation time by *B. subtilis* in SmF

of medium pH was shown after 60 h of fermentation which resulted in the decrease of amylase production by *B. subtilis*. The decrease of amylase production might due to the metabolic activity of bacteria which was relatively sensitive to the rising of medium pH. Indeed, Vijayabaskar et al. [25] showed that at higher medium pH, the metabolic activity of the bacteria would be suppressed and thus, inhibiting the enzyme production. On the other hand, Gupta et al. [26] reported that most *Bacillus* strains used commercially for the production of bacterial amylase under SmF have the optimum pH between 6.0 and 7.0 for growth and enzyme production.

3.4 Cellulase Production by *B. subtilis*

In this study, *B. subtilis* was observed to produce extracellular cellulase using barley husk as the substrate under SmF. The results illustrated in Fig. 7 shows a positive correlation between the cell growth and cellulase production, followed by a parallel decline with the growth of *B. subtilis*. It is obvious to note that the cellulase production was coherent with the cell growth pattern where it increased with the increasing cell growth during the logarithm phase before entered to the death phase. Similarly, Damiano et al. [27] reported that the maximum cellulase productivity in various microorganisms was achieved during the early stationary phase of growth, but declining thereafter during the death phase. Surprisingly, for the cellulase production, there was observed increment of activity with 6.909 fold from 0.044 ± 0.011 U/mL at 12 h to 0.304 ± 0.008 U/mL, producing $9.53 \times 10^8 \pm 4.99 \times 10^7$ cells/mL at 48 h followed by enormous decline of activity to 0.091 ± 0.009 U/mL at 60 h of SmF. Obviously, cellulase production took place during logarithm phase from 12 h to 48 h. The incubation period to achieve the maximum peak cellulase activity by *B. subtilis* was occurred at 48 h which yielded 0.304 ± 0.008 U/mL with its productivity of 18.23 U/mL/h. Similarly, a study by Bansal et al. [16] reported that cellulase synthesis started during the logarithm phase of growth with exhibiting the highest activity of 0.30 U/mL. Apparently, Fig. 7 also suggests the production of cellulase by *B. subtilis* in SmF was optimally expressed at the end of the exponential phase. The prolonged fermentation period after the optimum time course was generally accompanied by a decrease in the growth rate and cellulase productivity, which gradually declined to 0.006 U/mL by $9.08 \times 10^7 \pm 6.13 \times 10^6$ cells/mL

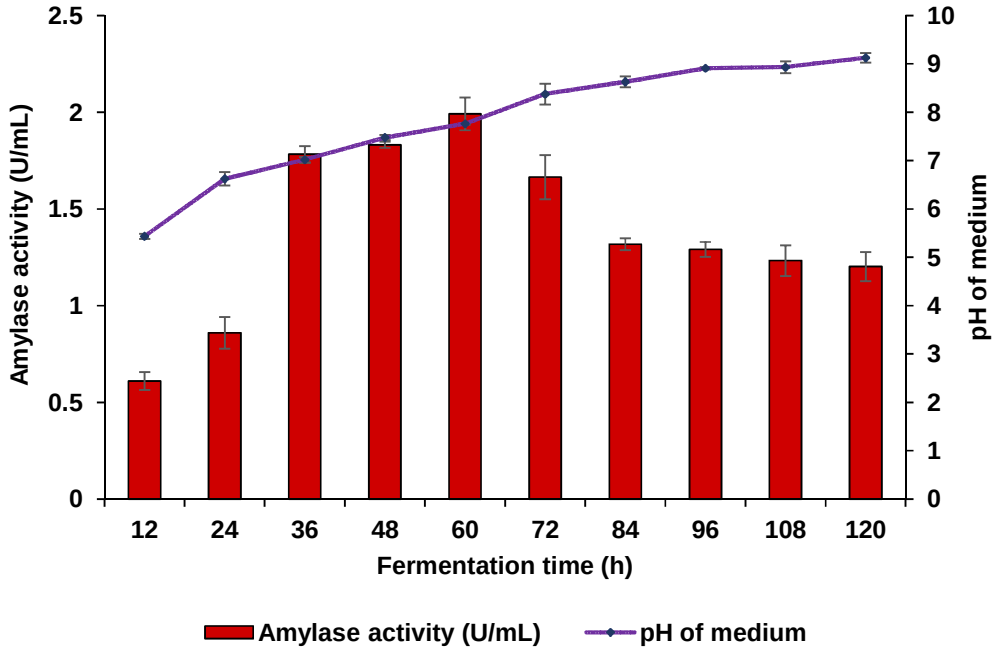

Fig. 6. Correlation of amylase activity with the pH of medium over fermentation time by *B. subtilis* in SmF

after 84 h of incubation. However, it was observed that there was no possible significant cellulase production occurred as the fermentation prolonged beyond 84 h as shown in Fig. 7. It might due to the depletion of nutrients in the medium which stressed the bacterial physiology system resulting in the inactivation of secretary machinery of cellulase. In fact, a fairly common observation has been reported where *Bacillus* spp are lacking of their complete cellulase system. The main reason being endoglucanase which does not hydrolyse crystalline cellulose would trigger the cellulase production at certain time [28]. Fig. 8 shows that the relationship between the protein concentration and cellulase activity was parallel. The cellulase production increased with the protein concentration increased within 48 h. On the contrary, as the fermentation extended, the cellulase production decreased with the diminution of protein concentration. Certainly, the concentration of protein shows a positive relationship with the cellulase production. From the result, the highest activity of cellulase produced in SmF was achieved at 0.304 ± 0.008 U/mL with its specific cellulase activity of 0.73 U/mg at 48 h of fermentation. Apparently, there was no further production beyond the 84 h of SmF. Based on Fig. 9, the maximum cellulase activity of 0.304 ± 0.008 U/mL occurred at pH 7.48 at 48 h under SmF. Similarly, Ray et al. [29] reported that the maximum cellulase activity by *B. subtilis* CY5 and *B. circulans* TP3 were observed between pH 7.0 and 7.5, respectively. From the study, it was observed that during the first 12 h of SmF, there was a drop in the medium pH from 6.55 to an acidic pH of 5.43 ± 0.05 which as a result, generated the extreme low cellulase production of 0.044 ± 0.011 U/mL. When medium compositions and pH change, cellular enzyme is inactive in either acidic or alkaline growth conditions [24]. When medium pH is altered below or above the optimum cellulase activity, the activity would decrease and denature where the absolute low cellulase activity of 0.006 U/mL occurred at pH 8.63 ± 0.11 at 84 h of SmF in this study. There was no possible cellulase production occurred in more alkaline medium as fermentation prolonged beyond 84 h. Fig. 9 shows an increase in medium pH over fermentation time of *B. subtilis* where cellulase was inactive at higher medium pH. In fact, a study by Tizon et al. [30] reported that cellulase exhibited its optimal activity at medium pH 6.0 due to its stable crystalline structure at slight acidic pH. Consequently, there was no further cellulase production beyond 84 h due to the higher alkaline medium pH which was above pH 8.63 in this study.

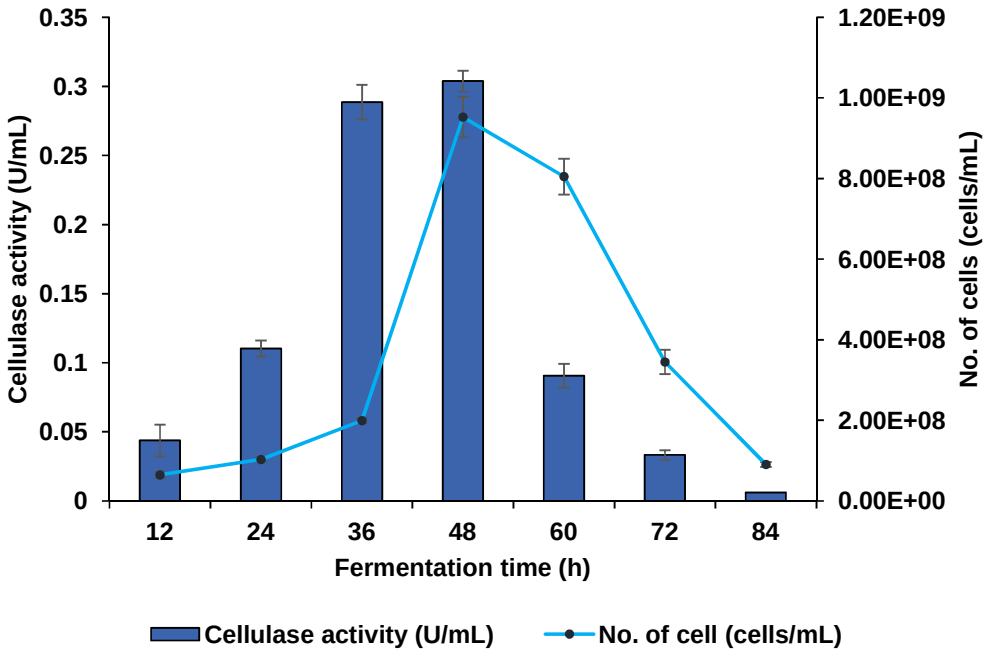

Fig. 7. Correlation of cellulase activity with the number of cells over fermentation time by *B. subtilis* in SmF

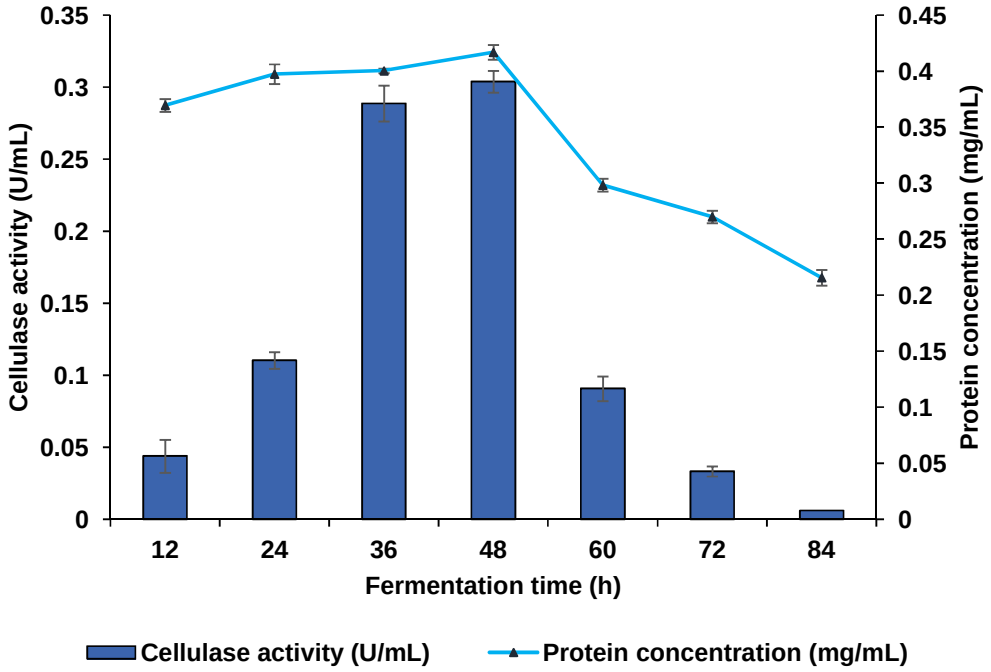

Fig. 8. Correlation of cellulase activity with the protein concentration over fermentation time by *B. subtilis* in SmF

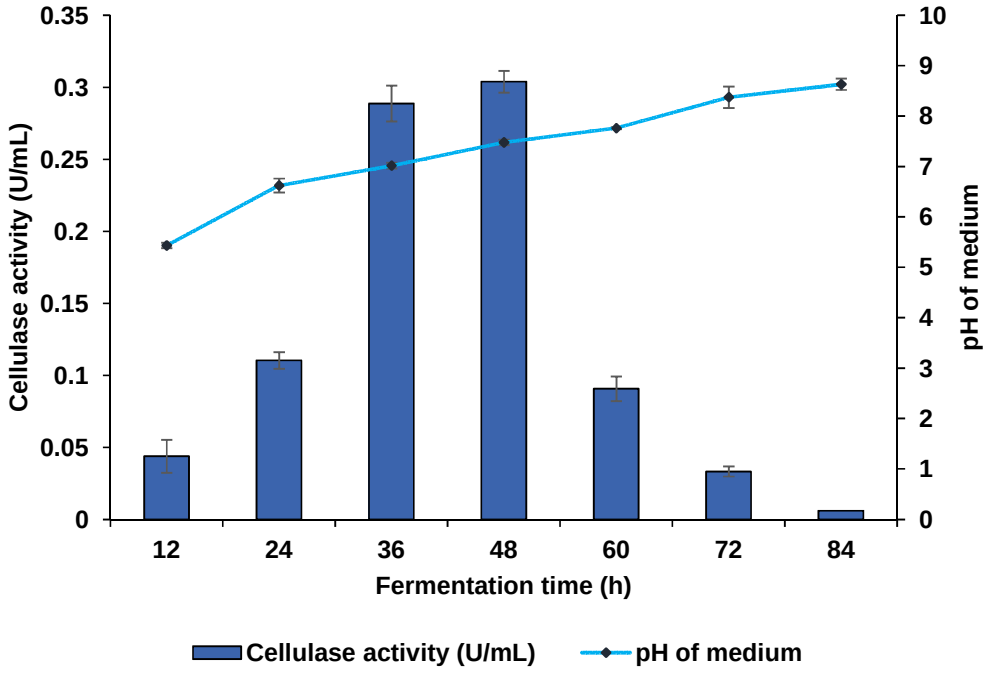

Fig. 9. Correlation of cellulase activity with the pH of medium over fermentation time by *B. subtilis* in SmF

3.5 Xylanase Production by *B. subtilis*

Besides amylase and cellulase, xylanase ativity assay was also carried out in this study. Fig. 10 shows the production of xylanase by *B. subtilis* in SmF under optimum growth conditions. In the presence of barley husk as the prime carbon source, growth and xylanase production continued to increase parallel up to 48 h of fermentation. Thereafter, even though, the cell growth ceased, enzyme production continued to increase even after 48 h of SmF. Based on the study, surprisingly, the xylanase activity increased by 89% from 0.696 ± 0.046 U/mL at 12 h to 1.311 ± 0.030 U/mL at 60 h of SmF. In fact, xylanase synthesis started during the logarithm phase and increased gradually during the incubation period, exhibiting the highest activity of 1.492 ± 0.039 U/mL with its productivity of 89.52 U/mL/h after 72 h of incubation, producing $3.45 \times 10^8 \pm 3.02 \times 10^7$ cells/mL. In literature review, xylanase production of 2.0 U/mL and 2.2 U/mL under SmF using kraft pulp and oat as their carbon sources were observed, respectively [31, 32]. Based on their results, the xylanase activity reached its maximum activity at 72 h of SmF. Bansal et al. [16] stated that the xylanase production of 95 U/mL was obtained at 72 h under the optimized SmF. Another study also agreed by Bajaj and Manhas [33]. Their study showed that the maximum xylanase activity was gained after 72 h of fermentation with the production of 29.7 U/mL. Furthermore, alkalophilic *Bacillus* NT-9 produced the maximum xylanase activity after 72 h of fermentation [34]. Apparently, the increase in xylanase activity during the later stages of cell growth in the medium might due to the release of small amounts of xylanase from the aged cells entering into autolysis process [35]. After 72 h of SmF, xylanase production was acceptable with 1.468 ± 0.028 U/mL, but declined thereafter to reach the minimum level of 0.891 ± 0.024 U/mL at 120 h. It shows further incubation did not increase xylanase yield, but it resulted in a decline otherwise. Probably, the reduction in xylanase yield was due to proteolysis and depletion of available nutrients to the microorganisms [36]. Fig. 11 demonstrates the production of soluble protein by *B. subtilis* during xylanase production in SmF. It shows a correlation of soluble protein concentration and xylanase production. Based on the result obtained, soluble protein at the time of maximum xylanase activity of 1.492 ± 0.039 U/mL was achieved at 72 h under SmF. Additionally, specific xylanase activity of 6.81 U/mg of protein was occurred at 84 h. Xylanase activity of 1.468 ± 0.028 U/mL at 84 h was obtainable with the soluble protein concentration of 0.216 mg/mL of protein. Based on Fig. 12

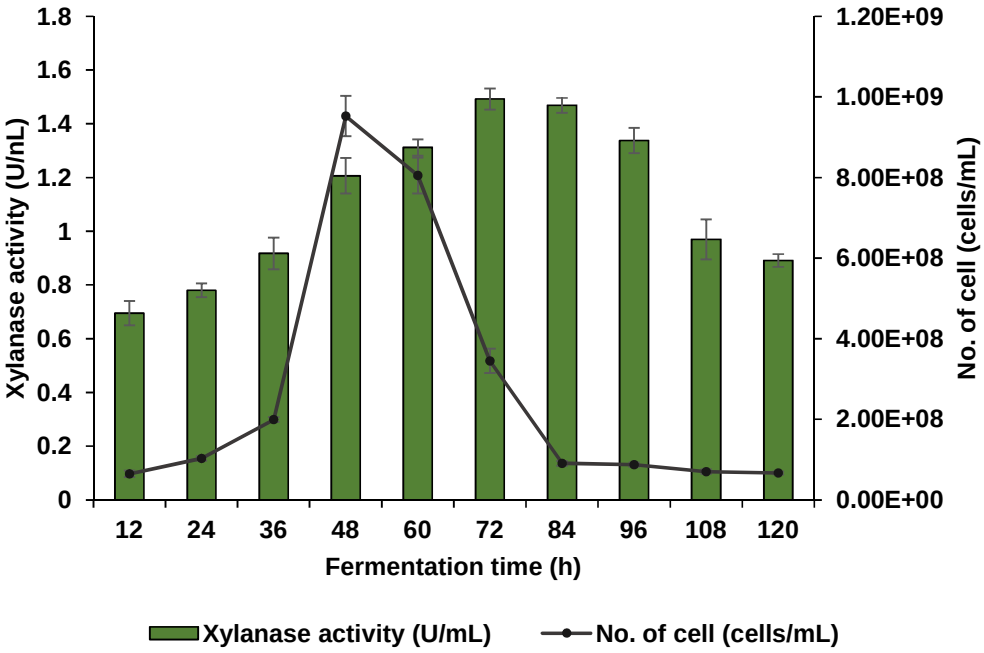

Fig. 10. Correlation of xylanase activity with the number of cells over fermentation time by *B. subtilis* in SmF

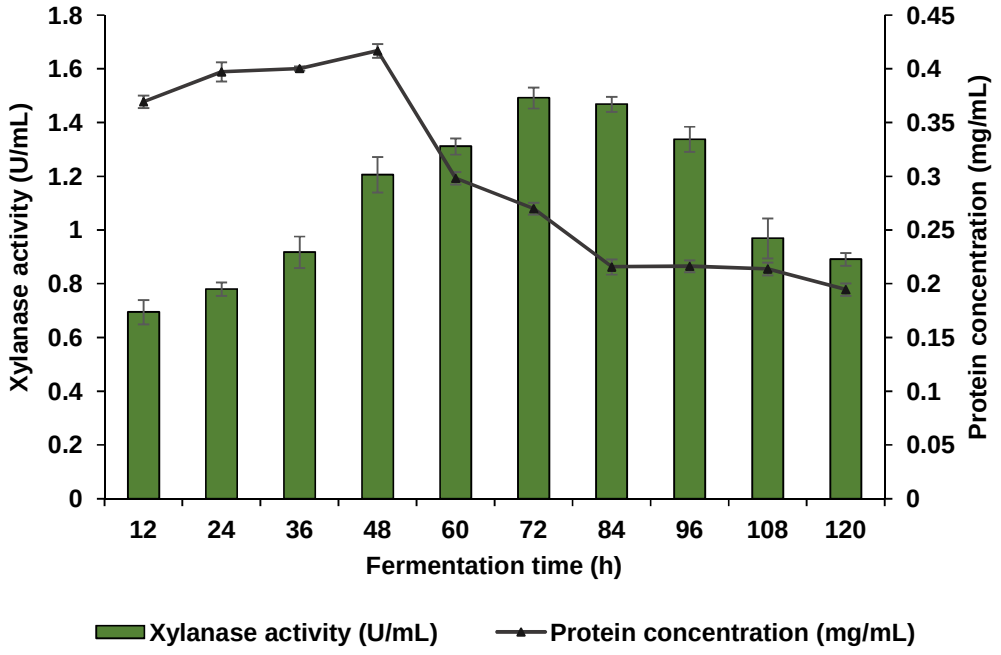

Fig. 11. Correlation of xylanase activity with the protein concentration over fermentation time by *B. subtilis* in SmF

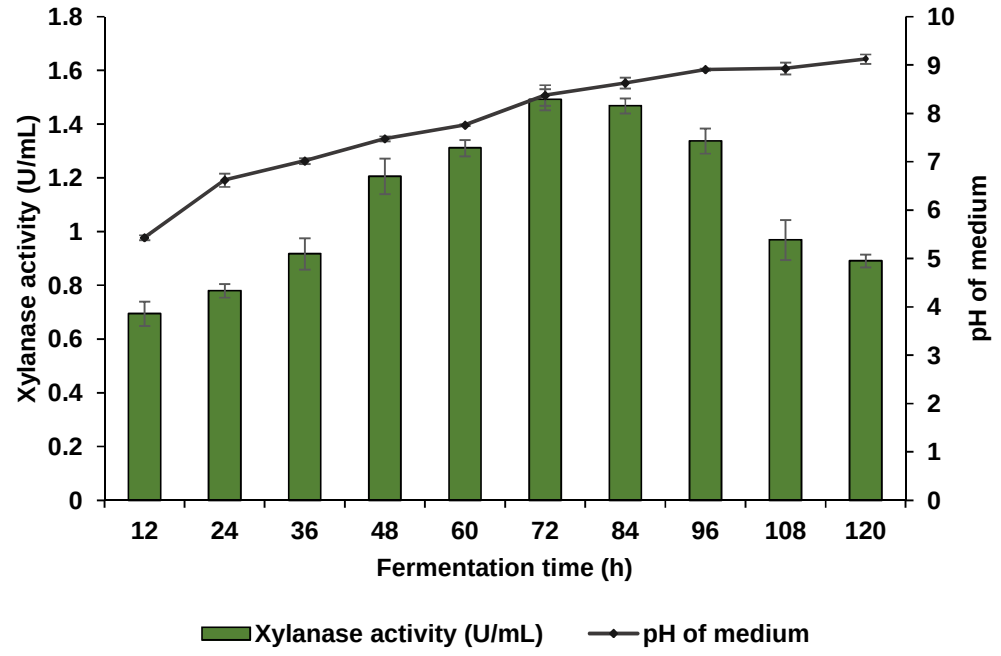

Fig. 12. Correlation of xylanase activity with the pH of medium over fermentation time by *B. subtilis* in SmF

the maximum xylanase activity of 1.492 ± 0.039 U/mL occurred at pH 8.38 ± 0.21 at 72 h under SmF. Correspondingly, Sepahy et al. [35] showed that the maximum xylanase production by *B. mojavensis* AG137 attained at medium pH 8. Another study also stated *Bacillus* NT 9 showed the maximal xylanase production at medium pH 8 [34]. From the result, beyond 72 h

of incubation, xylanase production was attainable producing 1.468 ± 0.028 U/mL at 84 h to 0.891 ± 0.024 U/mL at 120 h at medium pH 8.63 ± 0.11 and pH 9.13 ± 0.10, respectively. A study from Bajaj and Manhas [33] reported that *B. licheniformis* P11 (C) xylanase exhibited its maximum activity between pH 8 and pH 9. Moreover, acidic medium pH 4 to pH 6 generally favors fungal xylanases, whereas higher pH favors bacterial xylanases [35].

3.6 Summary of the Kinetic Study of Cell Growth and Enzymes Production by *B. subtilis* in SmF

B. subtilis is a general producer of amylase, cellulase and xylanase. The rate of synthesis for different enzymes are markedly dissimilar, where xylanase and amylase production were induced before cellulase. A kinetic approach was selected based on the theoretical understanding of the entire metabolic process to formulate suitable calculations to describe the production of biomass and enzymes under SmF. A summary of kinetic parameters of cell growth and enzymes production by *B. subtilis* is shown in Table 1. The kinetic of enzymes production compatible to Leudeking-piret model which defined by logistic growth model and Monod model. The specific growth rate (μ) defined as the velocity of biomass synthesis at a particular time interval related to the biomass concentration is expressed as (h^{-1}) [37]. There is a simple relationship between growth and generation time. From the results, *Bacillus* culture possessed the maximum specific growth rate (μ_{max}) of 0.55 h^{-1} during the phase of exponential growth with generation time of approximately 76.80 min under optimal batch growth condition. Different microorganisms possess different maximum specific growth rates. Based on the result findings, cellulase production was increased with the specific growth rate, approaching the maximum at the highest growth rates. Consequently, the maximum biomass concentration (X_{max}) calculated in the experimental data was $9.53 \times 10^8 \pm 4.99 \times 10^7$ cells/mL at 48 h with the maximum cell productivity of 1.98×10^{10} cells/L/h and growth coefficient of 7.96×10^8 cells/mg glucose. Enzymes productivity is analysed by assessing the biomass produced with enzymes synthesised by *B. subtilis*. In general, the incubation period is directly related to the production of enzymes and other metabolic processes. According to the results attained, enzymes that produced by *B. subtilis* in this study possess the highest activity of amylase with 1.991 U/mL, followed by xylanase activity with 1.492 U/mL and lowest in cellulase activity of 0.304 U/mL. A study from Subramaniyan and Prema [38] mentioned that little is known about the relationship between cellulolytic and xylanolytic activity produced by *Bacillus* spp. Nevertheless, higher xylanase activity with poorer cellulase activity by *B. subtilis* occurred is desirable as shown in this study. Hence, based on the present findings, the cellulase productivity is relatively low compared to activity of amylase and xylanase. In this study, the maximal cellulase production was triggered during the post exponential growth phase that tend to decline as the bacteria culture entered to the death phase. The cellulase production is growth associated and the rate of product formation is directly proportional to the rate of biomass formation which is induced by the presence of the substrate in the medium. However, the rate of production of amylase and xylanase by *B. subtilis* in SmF is partly growth associated that directly proportional to the concentration of biomass. Their maximal enzymes production were formed after logarithm phase of growth was over where the cells were still metabolically active. Meanwhile, these results are in agreement with the reports from Al-Qodah et al. [39] and Akcan et al. [23] on the relationship between the pattern of cell growth and amylase production. On the other hand, the purity of enzymes is very significant during enzymes extraction. This can be measured by specific enzyme activity which is a term used in measuring the enzyme kinetics. There was a variable relationship between the protein concentration and specific enzyme function. In this study, the specific amylase activity of 6.68 U/mg with the specific cellulase activity of 0.73 U/mg and specific xylanase activity of 6.81 U/mg were attained. The larger the specific enzymes activity, the purer the enzymes produce. Based on these results, *B. subtilis* produces purer xylanase per mg protein in this study. Besides that, relatively high maximum amylase coefficient of 1.744 U/mg glucose was attained compared to xylanase coefficient of 1.540 U/mg glucose and cellulase coefficient of 0.25 U/mg glucose. On the other hand, the medium pH also serves as a valuable indicator for the successful enzyme synthesis [40]. Indeed, the changes of medium pH observed during the growth of microorganisms would affect product stability in the medium [26]. Based on the result findings, the maximum growth of *B. subtilis* and cellulase production occurred at pH 7.48, maximum amylase production at pH 7.76 whereas maximum xylanase activity found at pH 8.38.

Table 1. Kinetic of cell growth and enzymes production by *B. subtilis* in SmF

Cell growth parameter	Cell growth		
Fermentation time at the maximum cell concentration (h)	48		
Optimum medium pH	7.48		
Maximum cell concentration, X_{max} (cells/mL)	9.53×10^8		
Maximum specific growth rate, μ_{max} (h^{-1})	0.5451		
Generation time, Td (min)	76.80		
Cell productivity (cells/L/h)	1.98×10^{10}		
Growth coefficient (cells/mg glucose)	7.96×10^8		
Enzymes production parameter	**Amylase**	**Cellulase**	**Xylanase**
Fermentation time at the maximum enzyme activity (h)	60	48	72
Optimum medium pH	7.76	7.48	8.38
Maximum enzyme production, P_{max} (U/mL)	1.991	0.304	1.492
Specific enzyme activity (U/mg protein)	6.68	0.73	6.81
Enzyme productivity (U/mL/h)	119.48	18.23	89.52
Enzyme coefficient (U/mg glucose)	1.744	0.25	1.540

The maximal enzymes production is favour at slight higher medium pH in this study. This might due to the composition of cell wall and plasma membrane of microorganisms that are affected by the culture medium pH. The changes of the initial medium pH might lead to change of the nature of cell wall and cell membrane hence, affecting growth of *B. subtilis* and enzymes production [41]. In consequence, maximal enzymes production is generally thrived at different optimum culture medium pH and thereafter, it declines beyond the optimum medium pH.

4. CONCLUSION

In conclusion, the results obtained in the current study demonstrate that *B. subtilis* is a potential producer of amylase, cellulase and xylanase which possess many applications in biotechnology. Maximum enzymes production was generated using barley husk which made the whole fermentation process economically viable. A kinetic study of the batch fermentation process was used to describe the growth of *B. subtilis* and production of enzymes. The different values of the various kinetic parameters such as the maximum specific growth rate, generation time, cell productivity, specific enzymes productivity and enzymes productivity were calculated using experimental data. Information about the relationship between the growth and enzyme kinetics is relatively crucial in the design and development of an efficient bioprocess technology. From the result findings of the current study, the enzymes produced by *B. subtilis* that possessed the highest activity and productivity was amylase with 1.991 U/mL and 119.48 U/mL/h, followed by xylanase with 1.492 U/mL and 89.52 U/mL/h, and lastly, cellulase with the lowest activity and productivity of 0.304 U/mL and 18.23 U/mL/h, respectively. Cellulase productivity was relatively low compared to amylase and xylanase. The production of cellulase was directly related to the growth of the bacterial. The maximum cellulase activity was obtained at the end of the exponential growth phase. Yet, the production of amylase and xylanase were partly growth associated where their maximal production were formed after the exponential phase of the growth was over.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Kohli D, Garg S, Jana AK. Thermal and morphological properties of chemically treated barley husk fiber. International Journal of Research in Mechanical Engineering and Technology. 2013;2:153-156.
2. Ho HL, Ajounmah MC. Overproduction of xylanase from mutants of *Bacillus subtilis* with barley husk as the prime carbon source under submerged fermentation after random mutagenesis using ethyl methane sulfonate (EMS) and acridine orange (AO). British Microbiology Research Journal. 2016;14(1):1-17.
3. Haltrich D, Nidetzky B, Kulbe KB, Steiner W, Zupancic S. Production of fungal xylanases. Bioresource Technology. 1996;58:137-171.

4. Bhatnagar D, Joseph I, Raj RP. Amylase and acid protease production by solid state fermentation using *Aspergillus niger* from mangrove swamp. Indian Journal of Fisheries. 2010;57(1):45-51.
5. Chinedu SN, Nwinyi OC, Okochi VI. Growth and cellulose activity of wild-type *Aspergillus niger* ANL301 in diferent carbon source. Canadian Journal of Pure and Applied Sciences. 2008;2:357-362.
6. Bailey MJ, Biely P, Poutanen K. Inter laboratory testing of methods for assay of xylanase activity. Journal of Biotechnology. 1992;23:257-270.
7. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry. 1951;193(1):265-275.
8. Thangam EB, Rajkumar GS. Studies on production of extracellular protease by *Alkaligenes faecalis*. World Journal of Microbiology Biotechnology. 2000;16:663-666.
9. Sanghi A, Garg N, Kuhar K, Kuhad RC, Gupta VK. Enhanced production of cellulase-free xylanase by alkalophilic *Bacillus subtilis* and its application in biobleaching of kraft pulp. BioResources. 2009;4(3):1109-1129.
10. Bozic N, Jordi RJ, Lopez-Santin J, Zoran VZ. Optimization of the growth and α-amylase production of *Bacillus subtilis* IP 5832 in shake flask and laboratory fermenter batch cultures. Journal of the Serbian Chemical Society. 2011;76(7):965-972.
11. Budde I, Steil L, Scharf C, Volker U, Bremer E. Adaptation of *Bacillus subtilis* to growth at low temperature: A combined transcriptomic and proteomic appraisal. Microbiology. 2006;152(3):831-853.
12. Ho HL. Batch submerged fermentation in shake flask culture and bioreactor: Influence of different agricultural residuals as the substrate on the optimization of xylanase production by *Bacillus subtilis* and *Aspergillus brasiliensis.* Journal of Applied Biotechnology and Bioengineering. 2016;1(3):00016.
13. Abdelwahed NAM, El-Naggar NE, Saber WIA. Factors and correlations controlling cellulase-free xylanase production by *Streptomyces Halstedii* NRRL B-1238 in submerged culture. Australian Journal of Basic and Applied Sciences. 2011;5(10): 45-53.
14. Olkku J, Kotaviita E, Salmenkallio-Marttila M, Sweins H, Home S. Connection between structure and quality of barley husk. Journal of the American Society of Brewing Chemists. 2005;63:17-22.
15. Swinnen IAM, Bernaerts K, Dens EJJ, Geeraerd AH, Van-Impe JF. Predictive modelling of the microbial lag phase: A review. International Journal of Food Microbiology. 2004;94(2):137-159.
16. Bansal N, Soni R, Janveja C, Soni SK. Production of xylanase-cellulase complex by *Bacillus subtilis* NS7 for the biodegradation of agro-waste residues. Lignocellulose. 2012;1(3):196-209.
17. Logan NA, de Vos P. Bacillus. In: Bergey's Manual of Systematic Bacteriology: The Firmicutes" (De Vos P, Garrity G, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH, Whitman WB, eds.). Springer-Verlag, New York, USA. 2009;21-128.
18. Vijayalakshmi, SK, Abha S, Chander P. Isolation and characterization of *Bacillus Subtilis* KC3 for amylolytic activity. International Journal of Bioscience, Biochemistry and Bioinformatics. 2012; 2(5):336-341.
19. Wilks JC, Kitko RD, Cleeton SH, Lee GE, Ugwu CS, Jones BD, BonDurant SS, Slonczewski JL. Acid and base stress and transcriptomic responses in *Bacillus subtilis*. Applied and Environmental Microbiology. 2009;75:981-990.
20. Ozdemir S, Matpan F, Guven K, Baysal Z. Production and characterization of partially purified extracellular thermostable alpha-amylase by *Bacillus subtilis* in submerged fermentation (SmF). Preparative Biochemistry and Biotechnology. 2011; 41(4):365-381.
21. Grover A, Maninder A, Sarao LK. Production of fungal amylase and cellulase enzymes via solid state fermentation using *Aspergillus oryzae* and *Trichoderma reesei*. International Journal of Advancements in Research and Technology. 2013;2(8):108-124.
22. Vidyalakshmi R, Paranthaman R, Indhumathi J. Amylase production on submerged fermentation by *Bacillus* spp. World Journal of Chemistry. 2009;4(1):89-91.
23. Akcan N. High level production of extracellular α-amylase from *Bacillus licheniformis* ATCC 12759 in submerged fermentation. Romanian Biotechnological Letters. 2011;16(6):6833-6840.

24. Riaz N, Ikram-Ul-Haq, Qadeer MA. Characterization of α-amylase by *Bacillus subtilis.* International Journal of Agriculture and Biology. 2003;5(3):249-252.
25. Vijayabaskar P, Jayalakshmi D, Shankar T. Amylase production by moderately halophilic *Bacillus cereus* in solid state fermentation. African Journal of Microbiology Research. 2012;6(23):4918-4926.
26. Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B. Microbial α-amylases: a biotechnological perspective. Process Biochemistry. 2003;38:1599-1616.
27. Damiano VB, Bocchini DA, Gomes E, Da Silva R. Application of crude xylanase from *Bacillus licheniformis* 77-2 to the bleaching of eucalyptus kraft pulp. World Journal of Microbiological Biotechnology. 2003; 19(2):139-144.
28. Mawadza C, Hatti-Kaul R, Zvauya R, Mattiasson B. Purification and characterization of cellulases produced by two *Bacillus* strains. Journal of Biotechnology. 2000;83(3):177-187.
29. Ray AK, Bairagi A, Sarkar GK, Sen SK. Optimization of fermentation conditions for cellulase production by *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 isolated from fish gut. Acta Ichthyologica Et Piscatoria. 2007;37(1):47-53.
30. Tizon RU, Serrano AEJ, Trifalgar RFM. Effects of pH on amylase, cellulose and protease of the Angelwing clam, *Pholas orientalis.* European Journal of Experimental Biology. 2012;2(6):2280-2285.
31. Saleem M, Akhtar MS, Jamil S. Production of xylanase on natural substrates by *Bacillus subtilis*. International Journal of Agriculture and Biology. 2002;4(2):211-213.
32. Mullai P, Rene ER. A simple multi layered neural network model for predicting bacterial xylanase production by *Bacillus* sp. using avena sativa as substrate. Biotechnology. 2008;7:499-504.
33. Bajaj BK, Manhas K. Production and characterization of xylanase from *Bacillus licheniformis* P11(C) with potential for fruit juice and bakery industry. Biocatalysis and Agricultural Biotechnology. 2012;1(4):330-337.
34. Han X, Zheng LS, Xie Y. Study on screening and cultivation conditions xylanase producing alkalophilic bacteria. Journal of Natural Sciences. 2004;9:125-128.
35. Sepahy AA, Ghazi S, Sepahy MA. Cost-effective production and optimization of alkaline xylanase by indigenous *Bacillus mojavensis* AG137 fermented on agricultural waste. Enzyme Resource. 2011;17(11):844-853.
36. Kapoor M, Nair LM, Kuhad RC. Cost-effective xylanase production from free and immobilized *Bacillus pumilus* strain MK001 and its application in saccharification of Prosopis juliflora. Biochemical Engineering Journal. 2008;38(1):88-97.
37. Rodriguez-Fernandez DE, Rodriguez-Leon JA, de Carvalho JC, Sturm W, Soccol CR. The behavior of kinetic parameters in production of pectinase and xylanase by solid-state fermentation. Bioresource Technology. 2011;102(22):10657-10662.
38. Subramaniyan S, Prema P. Cellulase-free xylanases from *Bacillus* and other microorganisms. FEMS Microbiology Letters. 2000;183:1-7.
39. Al-Qodah Z, Daghstani H, Geopel P, Lafi W. Determination of kinetic parameters of alpha-amylase producing thermophile *Bacillus sphaericus*. African Journal of Biotechnology. 2007;6(6):699-706.
40. Gigras P, Sahai V, Gupta R. Statistical media optimization and production of ITS alpha amylase from *Aspergillus oryzae* in a bioreactor. Current Opinion in Microbiology. 2002;45:203-208.
41. El-Banna TE, Abd-Aziz AA, Abou-Dobara MI, Ibrahim RI. Production and immobilization of alpha-amylase from *Bacillus subtilis*. Pakistan Journal of Biological Sciences. 2007;10(2):2039-2047.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/20237