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by Isnaeni Isnaeni

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Bacillus licheniformis BT5.9 Isolated from Changar Hot Spring, Malang, Indonesia, as a Potential Producer of Thermostable α-amylase

[1]Darah Ibrahim, [1]Han Li Zhu, [1]Nuraqilah Yusof, [2]Isnaeni and [1]Lim Sheh Hong*

[1]Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia
[2]Faculty of Pharmacy, Universitas Air Langga, Surabaya, Indonesia

Abstrak: Sebanyak 34 pencilan bakteria telah diperolehi daripada sampel tanah yang diambil dari Changar Hot Spring, Malang, Indonesia. Daripada jumlah ini, 13 pencilan menghasilkan zon hidrolisis pada medium agar kanji-nutrien dan menghasilkan pelbagai amilase dalam medium cecair. Satu pencilan telah dipilih sebagai pengeluar amilase terbaik dan telah dikenal pasti sebagai *Bacillus licheniformis* BT5.9. Peningkatan keadaan kultur (pH awal medium 5.0, suhu penanaman 50°C, kelajuan pergolakan 100 rpm dan saiz inokulum sebanyak 1.7×10^9 sel/ml) memberikan penghasilan amilase yang tertinggi (0.327 U/ml).

Kata kunci: Amilase, Bakteria Termofilik, *Bacillus licheniformis*, Mata Air Panas

Abstract: A total of 34 bacterial isolates were obtained from soil samples collected from Changar Hot Spring, Malang, Indonesia. Of these, 13 isolates produced a zone of hydrolysis in starch-nutrient agar medium and generated various amylases in liquid medium. One isolate was selected as the best amylase producer and was identified as *Bacillus licheniformis* BT5.9. The improvement of culture conditions (initial medium pH of 5.0, cultivation temperature of 50°C, agitation speed of 100 rpm and inoculum size of 1.7×10^9 cells/ml) provided the highest amylase production (0.327 U/ml).

Keywords: Amylase, Thermophilic Bacteria, *Bacillus licheniformis,* Hot Spring

INTRODUCTION

Thermostable enzymes from microorganisms have found a number of commercial applications because of their overall inherent stability (Demirijan *et al.* 2001). The most widely utilised thermostable enzymes are amylases, which are used in the starch industry (Demirkan *et al.* 2005; Sarikaya *et al.* 2000). These amylases should be active at the high temperatures of gelatinisation (100°C–110°C) and liquefaction (80°C–90°C) to economise these processes (Reddy *et al.* 2003). The α–amylases (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) hydrolyse α-1,4 glucosidic linkages in starch and related substrates (Bolton *et al.* 1997). In addition to liquefaction and gelatinisation, brewing (Leveque *et al.* 2000) and sizing in textile industries also use amylases (Pandey *et al.* 2000). Furthermore, amylases are used as an additive in detergents to remove starch spots from textiles, and they can be used to form dextrin in baking and distilling (De Souza & Magalhaes 2010). Therefore, the thermostability of the amylases must be

*Corresponding author: limshehhong77@gmail.com

matched to their application. Based on this demand, there has been a need and continual search for more thermophilic microorganisms and thermostable amylases.

Although amylases can be derived from several sources, such as plants, animals and microorganisms, amylases from microbial sources generally meet the industrial demands. Screening for microorganisms with higher amylase activities could facilitate the discovery of novel amylases that are suitable for industrial application (Wanderley *et al*. 2004). Fermentation using thermophilic microorganisms has many advantages, including a reduction in cooling cost, better solubility of substrates and the reduced risk of microbial contamination. In this study, we isolated and identified a thermostable bacterial α-amylase from a hot spring soil sample. Furthermore, the physical parameter conditions for cultivation of the new thermophilic isolate, *Bacillus licheniformis* BT5.9, were optimised.

MATERIALS AND METHODS

Isolation of Amylase Producers

Samples of soils and sediments collected from Changar Hot Spring at Malang, Indonesia, were kept in containers under sterile conditions and brought back to the laboratory. Upon arrival at the laboratory, a ten-fold dilution was made by mixing 1.0 g of sample with 9 ml of sterile distilled water and vortexing the mixture. The samples were then serially diluted from 10^{-1} to 10^{-4} with sterile distilled water and were spread on nutrient agar plates. The inoculated plates were incubated at 50°C for 36 hours with daily observation of the plates. Single colonies of different morphological characteristics, such as the size, shape, colour, elevation and margin, were identified from different plates streaked with diluted samples. The purified colonies were then streaked on nutrient agar slants and incubated at 50°C for 36 hours.

Qualitative Screening for Amylase Producers on Solid Agar Medium

Each colony was streaked on a nutrient agar plate that contained 1% starch (starch-nutrient agar) and incubated at 50°C for 36 hours with daily observation. Single colonies that formed hydrolysis zones were identified as starch utilising isolates. To confirm amylase production from the isolates, a 0.5% (w/v) iodine solution was poured over them, and the formation of clear zones indicated amylase production.

Quantitative Screening for Amylase Producers on a Liquid Medium

Cultivation medium contained the following (g/l): soluble starch (10); peptone (20); $MgSO_4.7H_2O$ (1.0); Na_2HPO_4 (3); $FeSO_4$ (0.3) and NaCl (0.1). The medium was sterilised and cooled to room temperature (30±2°C). One loopful of purified bacterial culture was used to inoculate 100 ml of cultivation medium in a 250 ml Erlenmeyer flask. The inoculated flasks were incubated at 50°C and agitated at 100 rpm for 48 hours. The flasks were harvested at 6-hour intervals, and the culture medium was filtered through Whatman No. 1 filter paper (Whatman Ltd.,

Kent, England). The cell-free filtrate was used as the enzyme source for the assay of amylase activity, and bacterial growth was determined spectrophotometrically using Genesys uv (Spectronic Unicam, Texas, USA) at 610 nm (Ajayi & Fagade 2006). The experiments were conducted in triplicate, and the results were expressed as the mean values of the triplicate experiments.

Identification of the Selected Strains of Amylase Producers

For 16S rRNA analysis (Soumitesh *et al.* 2007; Tamura *et al.* 2007), 16S rRNA, which is 1.542 kb in length, was used as a primer source. 16S rRNA is highly conserved between different species of bacteria, as it is a component of the 30S small subunit of prokaryotic ribosomes. PCR product sequencing was performed by First BASE Laboratories Sdn. Bhd. (Shah Alam, Selangor, Malaysia), and the sequences were used for DNA homology searches of the GenBank database at National Center for Biotechnology Information (NCSI) website (http://www.ncbi.nih.gov).

A pure culture of BT5.9 was selected for its significant potential as an amylase producer. The colony morphology, such as form, elevation, margin, colour, odour and the colony surface when grown on starch-nutrient agar plate, of this strain was observed. Gram staining and biochemical tests were performed according to the methods described in Bergey's Manual of Systematic Bacteriology (Sneath *et al.* 1986). Finally, the strain was identified by 16S rRNA analysis.

Enzyme Production Medium

The selected isolate was used, and the enzyme production medium used consisted of the following (g/l): soluble starch (10); peptone (2.0); $MgSO_4.7H_2O$ (1.0); Na_2HPO_4 (3.0); $FeSO_4$ (0.03) and NaCl (0.1). One millilitre of inoculum (1 x 10^8 cells/ml) was added to 99 ml of the cultivation medium in a 250 ml Erlenmeyer flask, and the flasks were incubated at 50°C for 48 hours at 100 rpm agitation speed. At 6-hour intervals, the medium was filtered using Whatman No.1 filter paper. The cell-free filtrate was used for the amylase assay, and the bacterial growth was determined spectrophotometrically at 610 nm. The experiments were performed in triplicate, and the results were expressed as the mean values of three experiments.

Optimisation of Culture Conditions

To increase amylase production by *B. licheniformis* BT5.9, the following culture condition variations were evaluated: the initial medium pH ranged from 4 to 8, the incubation temperatures were between 40°C and 55°C, the agitation speeds ranged from 50 to 200 rpm and the inoculum sizes varied from 0.5 to 8 x 10^9 cells/ml. After 12 hours of cultivation, the media were filtered using Whatman No.1 filter paper. The cell-free filtrate was used for the amylase assay, and the bacterial growth was determined spectrophotometrically at 610 nm. The experiments were performed in triplicate, and the results were expressed as the mean values of three experiments.

Amylase Assay

The amylase activity was determined based on release of reducing sugars from soluble starch, which was measured using a modification of the dinitrosalicylic acid (DNS) method as previously described (Miller 1959). Briefly, 1 ml of soluble starch solution [1% (w/v) soluble starch in 0.05 M sodium citrate buffer at pH 5.9] was heated at 50°C for 10 minutes in a water bath. Then, 0.1 ml of crude enzyme was added to the substrate, which was incubated again at 50°C for 10 minutes with gentle shaking. The reaction was stopped by the addition of 2.0 ml of DNS reagent. The reaction mixture was heated at 100°C for 10 minutes and was then cooled down to room temperature and diluted with 16.9 ml of distilled water. The absorbance of the mixture was determined spectrophtometrically at 540 nm. The amylase activity was determined based on the glucose standard curve of amylase activity. One international unit (IU) of amylase activity is defined as the amount of enzyme required to liberate one micromole of reducing sugar per minute under the assay conditions.

RESULTS

Isolation and Selection of Thermostable Amylase Producing Bacteria

Thermophilic amylase-producing bacteria were successfully isolated from soil samples collected from Changar Hot Spring in Malang, Indonesia. Thirty four isolates were obtained and 13 isolates were found to produce a halo zone of hydrolysis from the quantitative screening. Qualitative screening was then performed on these 13 isolates using liquid cultivation.

Table 1 shows the four isolates that produced higher amylase activity among those tested. From the results obtained, isolate BT5.9, which produced the highest amylase activity (at 12 hours) with 0.330 U/ml, was selected for the optimisation process. The results also indicate that enzyme production was not related to growth.

Table 1: Profiles of bacterial growth and amylase production by *B. licheniformis* BT5.9 after optimisation of culture conditions.

Isolates	Diameter of zone of hydrolysis (mm)	Activity of amylase at 12 hours cultivation (U/ml)	Growth of bacterial isolates at 12 hours cultivation (OD 610 nm)
BT 2.4	20±2.0	0.309±0.11	2.118±0.27
BT 3.5	18±2.0	0.109±0.09	1.643±0.16
BT 4.6	20±2.0	0.110±0.02	0.874±0.12
BT 5.9	22±2.7	0.330±0.05	2.016±0.38

Note: OD-optical density

Identification of Potential Amylase Producer Isolate BT5.9

The BT5.9 isolate was identified based on its microscopic characteristics, and the results are tabulated in Table 2. This isolate is a Gram positive rod-shaped

bacteria that is motile and produces polar endospores. The colony was pale in colour and produced a particular odour. Additionally, it was identified based on its biochemical and culture characteristics, as shown in Table 3. Isolate BT5.9 had positive results for the Voges-Prokaeur test and for the citrate and nitrate reduction tests, and it produced negative results for the indole, tyrosine and urease utilisation tests. The isolate was β–hemolytic on blood agar and grew well in medium containing 7% sodium chloride. Based on the results obtained, isolate BT5.9 was identified as a *Bacillus* sp.

Table 2: Microscopic characteristics of the isolate BT5.9.

Characteristics	Results
Colony	Irregular
Margin	Irregular
Elevation	Flat
Surface	Shiny and moist
Colour	Pale
Odour	Yes
Gram staining	Positive
Shape of vegetative cells	Rod shaped
Endospore formation	Yes
Motility	Actively motile

Table 3: Biochemical and culture characteristics of the isolate BT5.9.

Biochemical tests	Results
Growth	Aerobic and facultative anaerobic growth
Indole production	Negative
Voges-Proskaeur test	Negative
Catalase production	Positive
Citrate production	Positive
Oxidase production	Negative
Urease test	Negative
Nitrate reduction test	Positive
Hydrolysis of tyrosine	Negative
Hemolysis on blood agar plate	β-hemolytic
Growth in 7% sodium chloride	Positive
Growth at 55°C	Positive

Further identification was then performed by 16S rRNA gene analysis. A series of nucleotide sequen with 1433 bases was obtained (Fig. 1). The sequence was then used in a Basic Local Alignment Search Tool (BLAST) search

of GenBank, and the results had 99.86% similarity with *B. licheniformis*. A phylogenetic tree was constructed based on the aligned sequences of the genus *Bacillus* using the neighbour-joining method. According to the phylogenetic tree, *Bacillus* sp. BT5.9 was closely related to *B. licheniformis* ATCC 14580T (Fig. 2). Therefore, based on the results obtained, it was suggested that the BT5.9 isolate can be designated as *B. licheniformis* with the strain name of BT5.9.

Figure 1: Full-length sequence (1433 bp) of isolate BT5.9 or S1 (16S rRNA analysis).

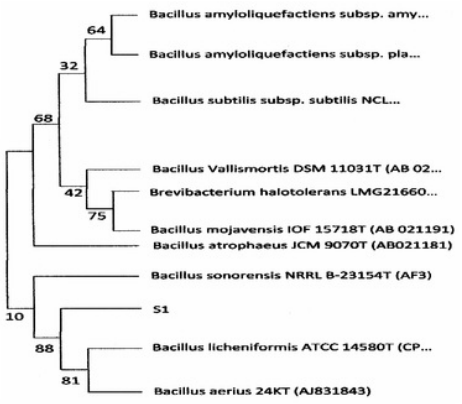

Figure 2: Evolutionary relationship of isolate BT5.9 or S1 with 10 related taxa.

Optimisation of Culture Conditions for Amylase Production by Isolate *B. licheniformis* BT5.9

Effect of initial medium pH

Figure 3 shows the effect of initial medium pH on amylase production and bacterial growth. The results showed that the highest amount of amylase was produced at an initial pH of 5.0 with 0.111 U/ml and that other pH values yielded lower enzyme production. In terms of bacterial growth, the highest bacterial growth achieved was 2.712 [optical density (OD) 610 nm] at an initial medium pH of 6.0. Again, a higher or lower pH value than 6.0 produced lower bacterial growth. It was observed that the highest amylase production occurred at pH 5.0. However, the highest bacterial growth occurred at pH 6.0, suggesting that the bacteria need an acidic environment to synthesise the enzymes and that synthesis was not growth dependent.

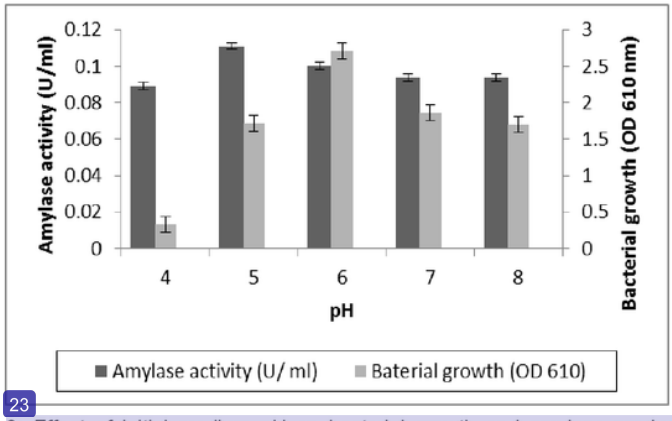

Figure 3: Effect of initial medium pH on bacterial growth and amylase production by *B. licheniformis* BT5.9.

Effect of cultivation temperature

Figure 4 shows that enzyme production gradually increased from 40°C and reached the optimal cultivation temperature at 50°C, with a maximum yield of 0.089 U/ml; additionally, amylase activity decreased in cultivation temperatures above 50°C. The growth of this strain reached its maximum rate when cultivated at 50°C (1.843 at OD 610 nm); however, it was observed that the enzyme production was not growth dependent in this study (Fig. 4). Because the isolate is thermophilic, it needs higher temperatures (45°C–55°C) to grow and produce the enzyme. Therefore, 50°C was used as the cultivation temperature in the subsequent experiments.

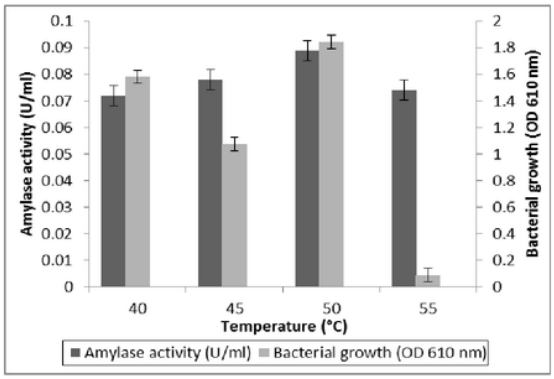

Figure 4: Effect of cultivation temperature on bacterial growth and amylase production by *B. licheniformis* BT5.9.

Effect of agitation speed

The effect of various agitation speeds (Fig. 5) shows that the maximal amylase production (0.094 U/ml) was achieved at 100 rpm and that other agitation speeds reduced amylase production. The results also indicated that the maximal growth of 3.859 (OD 610 nm) was achieved at 100 rpm. Agitation speeds that were higher or lower than 100 rpm resulted in decreased enzyme production and less bacterial growth. Although it appears that enzyme production is growth dependant, there is a trend toward growth association in the fermentation system. Therefore, an agitation speed of 100 rpm was used in the subsequent experiments.

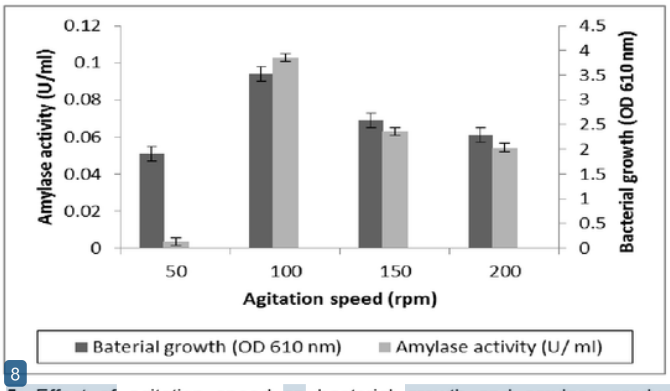

Figure 5: Effect of agitation speed on bacterial growth and amylase production by *B. licheniformis* BT5.9.

Effect of inoculum sizes

As shown in Figure 6, the results indicate that the optimal inoculum size was 1.7×10^8 cells/ml, as this size produced the highest amylase activity of 0.180 U/ml. The optimal inoculum size enabled maximal bacterial growth, at an OD of 2.06 at 610 nm. Higher or lower inoculum sizes than 1.7×10^8 cells/ml reduced amylase production. Therefore, an inoculum size of 1.7×10^8 cells/ml was used in the subsequent experiments.

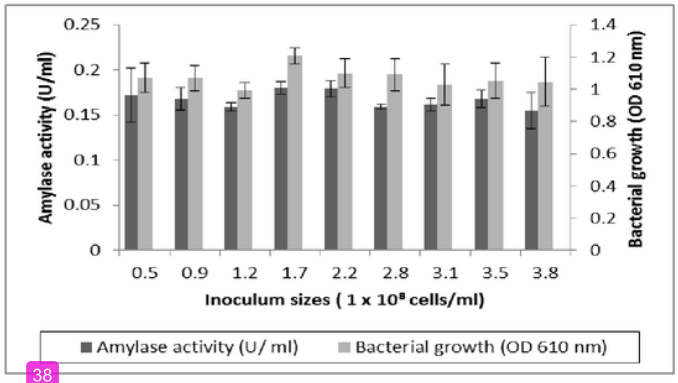

Figure 6: Effect of inoculum sizes on bacterial growth and amylase production by *Bacillus licheniformis* BT5.9.

Time Course Profile of Amylase Production and Bacterial Growth after Improvement of Cultural Conditions

Figure 7 shows the amylase production and bacterial growth by *B. licheniformis* BT5.9 after the optimisation of cultural conditions (initial medium pH of 5.0, cultivation temperature of 50°C, agitation speed of 100 rpm and inoculum size of 1.7×10^8 cells /ml). The results showed that the highest production of amylase, i.e., approximately 0.327 U/ml, was achieved at 12 hours of cultivation and that the enzyme production dropped thereafter. In terms of bacterial growth, the biomass increased gradually and achieved the highest growth at 12 hours of cultivation (1.789, OD 610 nm), but it then increased slowly until the end of cultivation time (48 hours) with an OD of approximately 2.140 at 610 nm.

DISCUSSION

A total of 34 isolates were obtained from 6 different samples, and 13 (38.23%) of these isolates were potential amylase producers. After performing a secondary screening process, which involved liquid fermentation, isolate BT5.9 was selected as the best amylase producer. This isolate was further identified and confirmed as *B. licheniformis* BT5.9, which is a gram positive rod-shaped cells that are capable of producing endospores. The soil is a vital natural medium that varies in

physico-chemical composition in different ecological zones. The presence of different microorganisms and their survival in soil can be justified by variations in soil composition. Organic matter in the soil serves as the sole substrate for microorganisms. Therefore, soil is an important biotope for identifying new industrial microorganisms. The presence of amylolytic microorganisms, especially among bacteria in soil samples, is usually linked to the presence of starch granules (Fossi *et al*. 2005; Muralikrishna & Nirmala 2005).

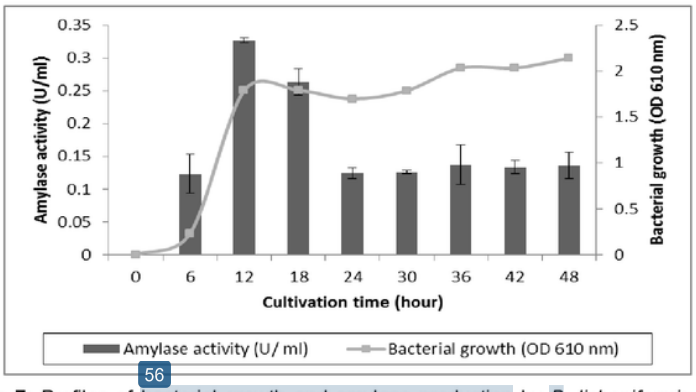

Figure 7: Profiles of bacterial growth and amylase production by *B. licheniformis* BT5.9 after improvement of culture conditions.

Optimisation of Culture Conditions for Bacterial Growth and Amylase Production by *B. licheniformis* BT5.9

The main limitation in the development of applications for thermostable amylases is the low production level of amylases by microorganisms. Therefore, a method for improving the production of this enzyme in submerged cultivation is required for bulk production and can enhance the enzyme's industrial applicability. Improvements of physical fermentation conditions (culture conditions) are important in the development of fermentation processes (Darah & Ibrahim 1996).

As a measurement of the hydrogen activity of a solution, pH is defined as the negative logarithm of the hydrogen ion concentration, and it can dramatically affect microbial growth. Each species of microorganism has a definite pH growth range and a pH for optimal growth. Although microorganisms often grow over a wide range of pH values, there are limits to their tolerance. Drastic variations in pH can harm microorganisms by disrupting the plasma membrane or inhibiting the activity of enzymes and membrane transport proteins. Changes in the external pH might also alter the ionisation of nutrient molecules and thus reduce their availability to the organism (Prescott 1990). Reddy *et al*. (2003) reported that the optimal pH for most amylases ranges from 4.0 to 11.0.

Microorganisms, like all other living things, are profoundly affected by the temperature of their environment, as it greatly influences enzyme production and growth. Indeed, microorganisms are unicellular and poikilothermic; therefore,

their temperatures vary with that of the external environment (Maria *et al*. 2004). One of the most important factors regarding the effect of temperature on growth is the temperature sensitivity of enzyme production. At low temperatures, the growth rate is slow, and the enzyme production tends to be low. However, as the temperature increases, the growth rate will also increase, which will lead to increased enzyme production. Each microorganism has its own optimal temperature at which the maximum enzyme production occurs. In this study, the optimum temperature for amylase production by *B. licheniformis* BT5.9 was 50°C. Temperatures above or below 50°C resulted in poor enzyme production. The favourable growth temperature for *B. licheniformis* BT5.9 was between 45°C and 65°C. Although the optimum temperature for bacterial growth was 50°C, the enzyme could be stable at a higher temperature; however, enzyme production was low when the temperature was higher than 50°C. Under a non-optimal temperature, the bacteria had a longer lag phase before growth and enzyme production began. Furthermore, increasing in the temperature to approximately 65°C led to a rapid decrease in bacterial growth, which consequently decreased enzyme production. According to Vidyalakshmi *et al*. (2009), the growth temperature plays an important role not only in the growth of bacteria but also in enzyme production. A cultivation temperature beyond the optimal one caused a reduction in the catalytic rate of amylase, as either the enzyme or substrate became denatured and inactive.

It is well documented that the agitation of most bacteria, especially in shake flask cultures, results in morphological changes in the cells, mainly due to their pelleted forms. The morphological changes of *B. licheniformis* BT5.9 were found to affect its production of amylase. The maximum production of the enzyme was obtained when the bacteria was agitated at 100 rpm. An agitation speed higher than this resulted in reduced bacterial growth and the production of amylase. One possible explanation is that there were greater mechanical or shear forces at high agitation speeds, which led to a higher rate of cell destruction, thus lowering enzyme production (Venkatadri & Irvine 1990). Under these conditions, excess oxygen content did not reduce the damage caused by shear forces (Darah *et al*. 2011). Conversely, lower agitation speeds also resulted in poor growth and led to the decreased production of the enzyme. This may have been due to the low amounts of dissolved oxygen in the cultivation medium. Thus, it was demonstrated that agitation speed plays an important role in enhancing secretion because it alters cell permeability.

Inoculum size plays an important role in achieving maximum bacterial growth and amylase production by *B. licheniformis* BT5.9 in a shake flask system. Generally, inoculum size affects the morphology of the bacteria as well as their progression through growth phases. Therefore, it is important to select a suitable inoculum size with respect to the type and age of microorganisms to obtain a high yield of the desired products (El-Enshasy *et al*. 2000). Sangeetha *et al*. (2004) reported that the optimal inoculum size in a fermentation process should be identified because lower inoculum density may result in insufficient biomass; however, higher inoculum density may produce too much biomass, which would deplete the nutrients necessary for product formation.

The results of this study revealed that bacterial growth increased as the inoculum size increased until the bacteria achieved the optimal inoculum size and that growth decreased sharply with inoculum sizes that were greater than the optimal one. According to Mukherjee *et al.* (2008), an inoculum size greater than the optimal size resulted in a steady decline of enzyme activity and biomass, which might be due to the exhaustion of nutrients in the cultivation medium. Enzyme production usually attains its peak when sufficient nutrients are available to the bacteria. Conditions with an imbalance between nutrients and proliferating biomass resulted in decreased enzyme synthesis. This decrease in the bacterial growth is caused by the rapid growth of other microorganisms, which will affect the length of stationary phase. Additionally, a short stationary phase led to low enzyme productivity (Sabu *et al.* 2006).

CONCLUSION

The thermophilic bacterium, *B. licheniformis* BT5.9 was isolated from a sample collected from Changar Hot Spring, Malang, Indonesia. This new isolate was able to produce more amylase after the improvement of cultivation conditions. The optimal conditions were determined to be a 50°C cultivation temperature with 12 hours of cultivation time at an agitation speed of 100 rpm and an inoculum size of 1.7×10^9 cells/ml. These results also suggested that *B. licheniformis* BT5.9 is a potential thermophilic microorganism that produces thermostable amylase.

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