original research article

Scale-up thermostable α-amylase production in lab-scale fermenter using rice husk as an elicitor by *Bacillus licheniformis*-AZ2 isolated from Qinarje Hot Spring (Ardebil Prov. of Iran)

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List of nonstandard abbreviations:
16S rRNA: 16S ribosomal RNA
Agro-wastes: Agricultural- wastes
ANOVA: Analysis of Variance
CRD: Completely Randomized Design
DNS Reagent: 3,5- di nitro salicylic acid reagent
LB medium: Luria-Bertani medium
OFAT method: one-factor-at-a-time method
rpm: Revolutions per minute
SmF: Submerged fermentation
SSF: Solid state fermentation
STR: Stirred Tank Reactor
vvm: Volume of air per unit volume of liquid medium per minute

Abstract:

Background and purpose: *Amylases are commercially important enzymes with various biotechnological, clinical and medical applications. This study aimed at scaling up α-amylase production elicited by rice husk in stirred-fermenter using* Bacillus lichneniformis*-AZ2 isolated from Qinarje Hot Spring.*

Materials and methods: *Effect of temperature, aeration rate and agitation speed on bacterial growth and α-amylase production were investigated under batch fermentation process in a 3-Lit stirred-fermenter. OFAT method was followed to select optimum level of each parameter. Other factors were set upon the results of previous experiments carried out in shake-flask scale.*

Results: *Maximum α-amylase production of 17.66 ± 0.87 U/mL (2.1 folds more than shake-flask cultures) was achieved in stirred-fermenter with optimized agitation speed of 100 rpm and 1 vvm aeration rate at 37ºC after 60 h of incubation. This time was shorter than the corresponding fermentation time obtained from shake-flask experiments by half. A comparison of kinetic parameters of fermentation in stirred-fermenter and shake-flask cultures revealed that B. licheniformis-AZ2 was more active to synthesize α-amylase in fermenter. In shaken cultures Q_x, Q_p, $Y_{p/x}$, μmax, q_p and t_d, were 0.27 (g/L/h), 228.6 (U/L/h), 13.64 (U/g), 0.055 (h^{-1}), 0.76 (U/g/h) and 12.48 h, whereas in stirred-fermenter the above values were 0.40 (g/L/h), 723.1 (U/L/h), 45.17 (U/g), 0.120 (h^{-1}), 5.42 (U/g/h) and 5.78 h, respectively.*

Conclusions: *SmF in stirred-fermenter is a potential strategy for α-amylase production. Although for commercialization further studies are needed in pilot-scale. Rice husk as a low-cost agro-waste is preferable to use as the carbon and energy sources for maximum α-amylase production.*

INTRODUCTION

Nowadays industrially important enzymes are mainly produced by thermophilic microorganisms originated from different geothermal environments such as Hot Springs and marine hydrothermal vents *(1, 2)*. Thermophilic microorganisms are able to live with optimal temperature ranges from 40°C to 80°C *(3)*. Due to the optimal activity and

stability of the released enzymes by extremophiles under extreme conditions, new catalytic alternatives for current industrial applications have recently been offered by extremozymes *(4)*.

Among the various industrial enzymes, amylases (i.e. α-amylases, ß-amylases and glucoamylases) rank first in terms of their commercial exploitation, so approximately that they share 25–33% of the world enzyme market *(5–7)*. α-amylases (E.C. 3.2.1.1.) are extra-cellular, endo starch-degrading enzymes which catalyze breaking down the internal α-1,4-*O*-glycosidic bonds in starch to low molecular weight products, such as glucose, maltose and maltotriose units with the retention of α-anomeric configuration in them *(8)*.

α-amylases have a wide variety of applications in starch processing, food, animal feed, baking, alcohol fermentation, detergent, textile and paper industries *(9)*. They also have promising applications in medical, clinical and molecular biology fields as well *(5, 10)*. Industrial production of this enzyme is mainly carried out in submerged mode of fermentation using different types of microorganisms such as bacteria, fungi, yeasts, and actinomycetes *(11, 12)*. However, enzymes derived from fungal and bacterial sources are more favored for industrial applications *(13)*. Among them, bacterial α-amylases due to the advantages and several characteristics that they offer are more preferred over fungal amylases *(14)*.

Bacilli are used as bacterial workhorses in industrial microbial cultivations for the production of a variety of enzymes *(15)*. It is estimated that *Bacillus* sp. enzymes comprise about 50–60% of the total global enzyme market *(5)*. *B. subtilis*, *B. stearothermophilus*, *B. licheniformis* and *B. amyloliquifaciens* are known as the best α-amylase producers among *Bacillus* species *(12)*. Although the production of bacterial α-amylases has been thoroughly studied in submerged fermentation (SmF) and solid state fermentation (SSF), because of the greater control on environmental factors such as temperature, pH, etc. and ease of handling, SmF is more common *(13, 16)*.

In general, bioprocess development is categorized in three main scales i.e. laboratory scale, pilot plant and production plant, which shake-flask scale also can be added to this list *(17)*. One of the major concerns that researchers were faced with, was to find a way in order to facilitate scaling up the submerged fermentations from shake flask level to production scale *(18)*. Bioreactors (also known as fermenters) are regarded as the heart of the fermentation process, which greatly have helped overcome this issue *(19)*. Bioprocess scale-up involves a series of stages, these stages are flask level (50–1000 g working capacity), laboratory level (2–20 kg working capacity), pilot level (50–5000 kg working capacity) and production level (25–5000 tons working capacity) *(17)*.

The scale-up of microbial product formation from a shake flask to a fermenter comprises optimization of culture conditions in fermentation processes *(20)*. So far, stirred-tank reactors (STRs) have been the most common fermenters used at the laboratory scale for studying SmF. The major preferences of this kind of fermenter over the rest are their homogenous system, excellent mixing and reasonably good heat and mass transfer rates, ease of handling and cleaning, compatibility with different operational patterns like batch, fed-batch, continuous, cell recycle and greater control over important factors, such as temperature, pH, air supply, rpm, and foam *(21)*. The secretion of microbial enzymes in fermentation process is dependent on several factors, such as composition of the fermentation medium, carbon and nitrogen sources, mineral salts, trace elements, type of strain, and also fermentation conditions i.e. pH, temperature, dissolved oxygen concentration, agitation etc. *(22, 23)*.

In stirred tank bioreactors aeration and agitation are two vital operational parameters, which play important role in scale-up of aerobic biosynthesis systems and industrial bioprocess development *(24)*. In aerobic fermentation, oxygen has an influence on enzyme secretion and this might be because of metabolic activities in the organism *(25)*. It has been recognized that amylase production by many *Bacillus* spp. is affected by the amount of dissolved oxygen. Therefore, forced aeration of the fermentation medium could be promising as it offers higher efficiency by combining aeration with agitation *(26)*.

In our previous study we screened several potent α-amylase producing bacteria isolated from Qinarje Hot Spring water sample, and found that *B. licheniformis*-AZ2 gives superior α-amylase activity. Then by implementing OFAT design α-amylase production was enhanced 2.4 folds in optimized fermentation medium compared to the basal medium. In addition, stability of the partially purified enzyme over a broad range of pH (6.0 to 10.0) and temperature (30°C to 80°C) in the presence of $CaCl_2$ implied that the produced calcium dependent enzyme could be categorized as alkaliphilic-thermostable α-amylases *(27)*. The present study intends to investigate the effect of incubation temperature, aeration rate and agitation speed on α-amylase production by *B. licheniformis*-AZ2 under Lab-scale fermenter in comparison with optimal conditions in shake flask.

MATERIALS AND METHODS

Chemicals and reagents

The DNS reagent and silicon antifoam reagent were prepared from Sigma-Aldrich (USA). Tryptone, peptone, tryptophan and bacteriological agar powder from HiMedia (India) were also used for experiments. All other chemicals were purchased from Merck (Germany).

Microorganism

The strain *B. licheniformis*-AZ2 used in the present study was obtained from Microbial Collection of Agricultural Biotechnology Department, at Bu-Ali Sina University, Hamedan, Iran. This strain has recently been isolated from Qinarje Hot Spring's water sample (82°C and pH 6.5) and identified based on 16S rRNA gene sequence method (GenBank: KT281607.1; https://www.ncbi.nlm.nih.gov/nuccore/893670518).

Inoculum development of B. licheniformis-*AZ2*

The strain of *B. licheniformis*-AZ2 was streaked on LB agar plate and incubated for 24 h at 40°C. Then, a loopfull of freshly grown bacterial cells were transferred into 100 mL of LB liquid medium [tryptone 10, NaCl 10 and yeast extract 5 (g/L)] and incubated at 40°C. Inoculum was taken from the early exponential phase of growth after 24 h.

α-Amylase production in shake flask

α-amylase production under submerged fermentation (SmF) condition was carried out in 250 mL Erlenmeyer flasks by taking 100 mL of optimized fermentation medium consisting of (g/L): rice husk 10, tryptone 10, tryptophan 1, $CaCl_2$ 0.3 and 2% (w/v) of Tween 80 (pH 9.0). The aliquot of 2% (v/v) of the total optimized medium was taken as inoculum. After inoculation, the culture flask of SmF was carried out using the optimum growth conditions at 40°C on a rotary shaker at 120 rpm for 144 h. This fermentation medium was previously optimized on a shake-flask scale using OFAT method for enhanced production of α-amylase by *B. licheniformis*-AZ2 *(27)*. The stimulatory effect of tryptophan was confirmed in complementary studies (Unpublished data).

α-Amylase production in stirred fermenter

Fermenter studies were carried out in a 3-L glass fermenter with a working volume of 1 L (Designed and constructed at Bu-Ali Sina University, Department of Agricultural Biotechnology). The fermentation medium was the same with shake flask culture. Agitation was performed using a flat-blade turbine impeller. The aeration system was an air filtered sterile (Membrane filter, 0.22 μm pore size) inlet through a ring sparger with portable air-flow meter. The pH of optimized fermentation medium was adjusted at 9.0 with 0.1 N HCl/ NaOH before sterilization at 15 lbs/in² pressure (121°C) for 20 min (Reyhan Teb, F2000, Iran). Foam was suppressed, when necessary, by the addition of sterilized silicon antifoam reagent (10 v/v). The pH was kept constant at 9.0 using sterilized solution of 0.1 N HCl/NaOH during entire fermentation process. The inoculated fermenter was incubated at 37°C for 96 h.

Optimization of cultural conditions in stirred fermenter

Experiments for optimization of the cultural conditions i.e. incubation temperature, agitation speed and aeration rate were conducted using OFAT methodology, the best choice in each experiment was used for the optimization of the next factor.

Incubation temperature

To study the effect of incubation temperature on α-amylase production and cell growth, the fermentation was carried out at different temperatures (30°C, 35°C, 37°C, 40°C, 45°C and 50°C) with constant aeration rate of 0.5 vvm and agitation speed of 100 rpm.

Aeration rate

The production of α-amylase was studied at aeration rates of 0, 0.5, 1.0, 1.5, 2.0 and 2.5 vvm. During the fermentation, the agitation speed of the impeller was kept at 100 rpm and optimal temperature of 37°C.

Agitation speed

Different agitation speeds of 50, 100, 150, 200, 250 and 300 rpm with optimal aeration rate of 1 vvm and incubation temperature of 37°C were investigated for optimum α-amylase production.

Statistical analysis

All experiments were carried out in three replications based on a completely randomized design (CRD). Mean values and standard errors were calculated from the average results of three fermenter runs for each process parameter. Statistical analysis was performed using SPSS V 16.0 software (SPSS Inc. Polar Engineering and Consulting, USA) based on single factor analysis of variance (ANOVA). Duncan's multiple range test was used for mean comparison of the treatments at a *p* value of 0.05.

Analytical methods

Determination of biomass

At regular intervals (6 h) samples from the fermenter and shake flasks were drawn. The total cell concentration was estimated by measuring optical density of fermented broth at 600 nm, and the obtained values were converted to cell dry weight (mg/mL) using a calibration curve. The cells were collected by centrifugation (10,000 ×g for 10 min at 4°C) in a refrigerated centrifuge (Eppendorf 5810R, Germany) and the cell free supernatant was analyzed for reducing sugar and enzyme activity.

Assay of α-amylase

α-amylase activity was determined by spectrophotometric method as described by Rick and Stegbauer *(28)*.
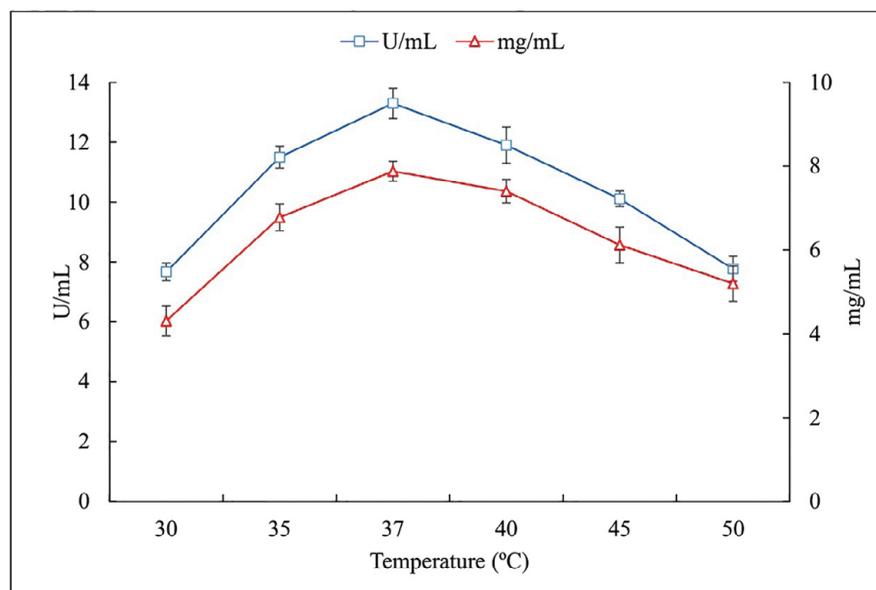
Figure 1. *Effect of temperature on cell growth and production of α-amylase by* B. licheniformis-*AZ2 at pH 9.0, aeration rate of 0.5 vvm and agitation speed of 100 rpm after 60 h.*
Mean ± S.E.M = Mean values ± Standard error of means of three replications.

According to the procedure α-amylase activity was assayed by adding 1 mL of enzyme (crude extract/fermented broth cell- free supernatant) to 1 mL of 1% (w/v) soluble starch in 50 mM Tris-HCl buffer (pH = 7.0) in a test tube. The test tubes covered and incubated for 5 min at 80°C in a water bath. Then 2 mL DNS reagent was added to each tube to stop the reaction and placed in boiling water bath exactly for 5 min. After cooling the samples in a cold water bath, the absorbance was read at 540 nm by spectrophotometer (Varian CARY 100 UV-VIS, Australia). An α-amylase activity unit was defined according to the amount of α-amylase required to catalyze the liberation of reducing sugar equivalent to one μ mole of maltose per minute under the assay conditions.

Kinetic analysis

Fermentation kinetic parameters were determined according to the methods described by Pirt *(29)* and Lawford and Rouseau *(30)*:

- μ_{max} was estimated as steepest slope of natural log (ln X) of biomass against time (h) of fermentation.

- t_d was calculated from μ_{max} by use of the following equation:

$$(1)\ t_d = 0.693/\mu_{max}$$

- Q_x (g cell mass/L/h) was calculated as highest slope of cell mass vs. time curve.

- Q_p (U/L/h) was estimated as maximum slope of culture medium's alpha amylase activity vs. time during the course of fermentation.

- $(Y_{p/x})$ Product yield coefficient (U/g), the value of $(Y_{p/x})$ was determined by the equation:

$$(2)\ Y_{p/x} = dp/dx$$

- (q_p) Specific product yield coefficient (U/g/h), the value of (q_p) was determined by the equation:

$$(3)\ q_p = Y_{p/x} \times \mu_{max}$$

RESULTS

Effect of incubation temperature

Optimal production of α-amylase in stirred fermenter was examined in different incubation temperatures from 30°C to 50°C with constant agitation speed of 100 rpm and aeration rate of 0.5 vvm. Figure 1 shows the effect of temperature on cell growth and enzyme production by *B. licheniformis*-AZ2 at different time courses. As can be seen, cell growth and enzyme production were increased as the incubation temperature was elevated from 30°C and reached maximum at 37°C both in enzyme production (13.30 ± 0.5 U/mL) and cell dry biomass (7.87 ± 0.24 mg/mL). At higher incubation temperatures α-amylase production by the cell was reduced significantly, which shows the inhibitory effect of higher temperatures on the growth of microorganism. Evaluation of kinetic parameters also revealed the same results as depicted in Figure 1. The product yield coefficient ($Y_{p/x}$), specific product yield coefficient (q_p), the volumetric rate of product formation (Q_p) and biomass formation (Q_x) were all maximum at 37°C showing optimum temperature for α-amylase production (Table 1).

Incubation Temperature (°C)	Q_x^1 (g/L/h)	Q_p^2 (U/L/h)	$Y_{p/x}^3$ (U/g)	μ_{max}^4 (h⁻¹)	q_p^5 (U/g/h)
30	0.21[d]	356.0[d]	14.09[e]	0.085[c]	1.20[d]
35	0.26[c]	391.6[c]	20.79[c]	0.096[ab]	2.01[c]
37	0.39[a]	544.0[a]	35.97[a]	0.102[a]	3.67[a]
40	0.31[b]	503.3[b]	32.78[b]	0.101[a]	3.31[b]
45	0.23[d]	324.0[d]	19.67[d]	0.092[b]	1.81[c]
50	0.22[d]	267.3[e]	13.97[e]	0.092[b]	1.29[d]

Kinetic parameters: ^1Q_x = g cell mass formation/L/h, ^2Q_p = Enzyme produced/L/h, $^3Y_{p/x}$ = Enzyme produced/g cell mass formation, $^4\mu_{max}$ (h⁻¹) = Specific growth rate, ^5q_p = Product formation specific rate U/g/h. Means with different letters indicate significant differences between treatments ($P < 0.05$, Duncan's multiple range test).

Effect of aeration rate and agitation speed

A basic requirement for a fermenter is the provision of aeration system that can maintain a high dissolved oxygen level for aerobic fermentation. In this connection different volume of air supply and rate of agitation speed were studied for the optimal enzyme production in stirred fermenter (Figure 2 and Figure 3).

The effect of aeration rate was investigated by comparing cell growth and enzyme production performances at aeration rates of 0, 0.5, 1, 1.5, 2 and 2.5 vvm with operating temperature and agitation speed maintained at 37°C

and 100 rpm, respectively. As depicted in Figure 2, the minimum level of cell growth and enzyme production were achieved when there was no air supplying, which shows lack of aeration during the fermentation was an extremely important limiting factor for cell growth and metabolite formation, so that increasing in the aeration rate from 0 to 1 vvm not only improved the cell growth but also enhanced the enzyme production level. As can be inferred from statistics, aeration (at 1 vvm) has also promoted drastic increases in the kinetic parameters of *B. licheniformis*-AZ2. Compared with no aeration fermentation, volumetric rates i.e. cell biomass formation and enzyme production were increased about 3.2 folds and 32 folds, while specific rate constants i.e. production yield coefficient ($Y_{p/x}$), and product formation specific rate (q_p) showed a 1152-fold, and 2353-fold increase in their values, respectively (Table 2).

The maximum α-amylase production (17.66 ± 0.87 U/mL), cell dry biomass (8.94 ± 0.25 mg/mL) and fermentation kinetic parameters were obtained when the aeration rate fixed at 1 vvm (Figure 2, Table 2). However, over aeration at 1 vvm caused a significant reduction in the cell growth and enzyme production. Thus aeration rate of 1 vvm was selected for further studies. Afterward different agitation speeds of 50, 100, 150, 200, 250 and 300 rpm were examined on α-amylase production at the constant temperature of 37°C and aeration rate of 1 vvm (Figure 3).

The highest α-amylase production (17.66 ± 0.87 U/mL; 16.45 ± 0.95 U/mL) and cell dry mass (8.94 ± 0.25 mg/mL; 8.50 ± 0.3 mg/mL) without any significant differences were achieved at agitation speeds of 100 rpm and 150 rpm, respectively. Whereas, beyond the optimal agitation speed range of 100 rpm to 150 rpm, cell growth
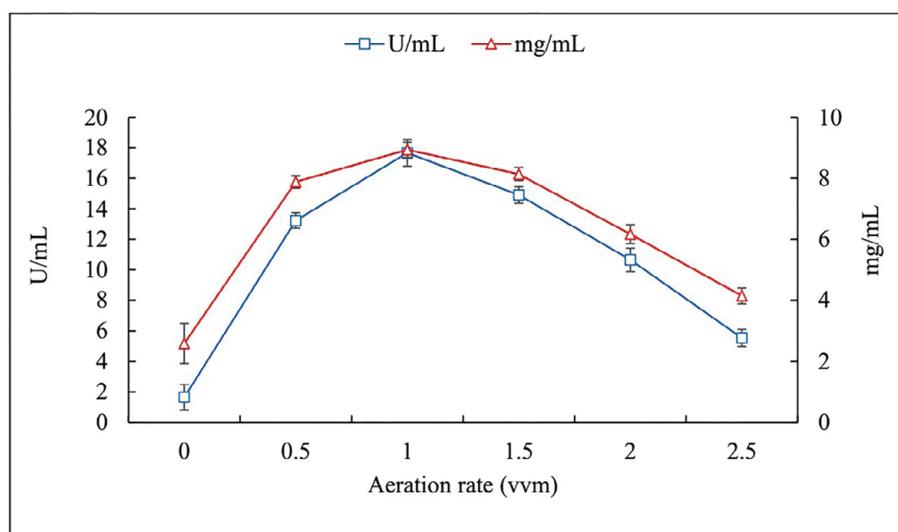
Figure 2. *Effect of aeration rate on cell growth and production of α-amylase by* B. licheniformis-*AZ2 at optimum temperature of 37°C, pH 9.0 and agitation speed of 100 rpm after 60 h.*
Mean ± S.E.M = Mean values ± Standard error of means of three replications.

Table 2. *Kinetic study of cell growth and α-amylase production by B. licheniformis-AZ2 at different aeration rates*

Aeration Rates (vvm)	Q_x[1] (g/L/h)	Q_p[2] (U/L/h)	$Y_{p/x}$[3] (U/g)	μ_{max}[4] (h^{-1})	q_p[5] (U/g/h)
0	0.13^d	22.6^d	0.04^f	0.059^d	0.002^f
0.5	0.39^a	544.0^b	35.97^b	0.102^{ab}	3.67^b
1	0.40^a	723.1^a	45.17^a	0.120^a	5.41^a
1.5	0.34^b	541.5^b	29.31^c	0.099^b	2.92^c
2	0.22^c	420.6^c	18.02^d	0.094^b	1.69^d
2.5	0.19^{cd}	138.6^e	6.31^e	0.078^c	0.55^e

Kinetic parameters: [1]Q_x = g cell mass formation/L/h, [2]Q_p = Enzyme produced/L/h, [3]$Y_{p/x}$ = Enzyme produced/g cell mass formation, [4]μ_{max} (h^{-1}) = Specific growth rate, [5]q_p = Product formation specific rate U/g/h. Means with different letters indicate significant differences between treatments ($P < 0.05$, Duncan's multiple range test).

Table 3. *Kinetic study of cell growth and α-amylase production by B. licheniformis-AZ2 at different agitation speeds*

Agitation Speed (rpm)	Q_x[1] (g/L/h)	Q_p[2] (U/L/h)	$Y_{p/x}$[3] (U/g)	μ_{max}[4] (h^{-1})	q_p[5] (U/g/h)
50	0.37^a	639.3^b	38.33^b	0.110^{ab}	4.43^{ab}
100	0.40^a	723.1^a	45.17^a	0.120^a	5.41^a
150	0.39^a	713.5^a	41.67^a	0.114^a	4.79^b
200	0.32^b	531.2^c	37.86^b	0.108^{ab}	4.10^c
250	0.27^c	442.0^d	24.57^c	0.092^b	2.27^d
300	0.26^c	387.8^e	16.66^d	0.082^b	1.36^e

Kinetic parameters: [1]Q_x = g cell mass formation/L/h, [2]Q_p = Enzyme produced/L/h, [3]$Y_{p/x}$ = Enzyme produced/g cell mass formation, [4]μ_{max} (h^{-1}) = Specific growth rate, [5]q_p = Product formation specific rate U/g/h. Means with different letters indicate significant differences between treatments ($P < 0.05$, Duncan's multiple range test).

and α-amylase production were lowered significantly. This may be due to adverse effect of high shear stress on the living cells. At agitation speeds ≥ 200 rpm, excessive foaming occurred thus the foams were suppressed by addition of silicon antifoam reagent.

The values of $Y_{p/x}$, q_p, Q_p and Q_x obtained after kinetic analysis of results revealed that the best aeration and agitation for α-amylase production were 1 vvm and 100 rpm, respectively (Table 2 and Table 3). Our results show that adjustment of agitation speed and aeration rate could greatly improve gas-liquid transfer and mixing in the fermenter.

Kinetics of cell growth and fermentation pattern of α-amylase in shake flasks and stirred fermenter under batch conditions

Figure 4 shows changes in dry cell biomass and α-amylase activity as a function of fermentation time in the shake flask and stirred fermenter. As shown, in the shake flask exponential growth began after a prolonged lag phase of 42 h and continued for 18 h until reaching stationary phase. The highest α-amylase production of 8.67 ± 0.5 U/mL and cell dry mass 6.41 ± 0.16 mg/mL were obtained in shake flask culture during the late stationary phase of growth after about 120 h. In contrast, in the stirred fer-
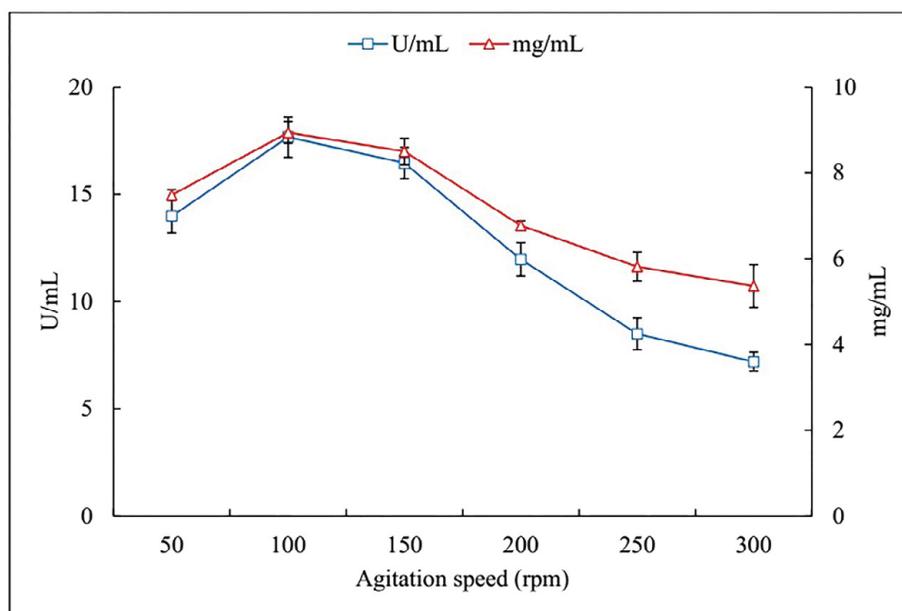
Figure 3. *Effect of agitation speed on cell growth and production of α-amylase by* B. licheniformis-AZ2 *at optimum temperature of 37°C, pH 9.0 and aeration rate of 1 vvm after 60 h.*
Mean ± S.E.M = Mean values ± Standard error of means of three replications.
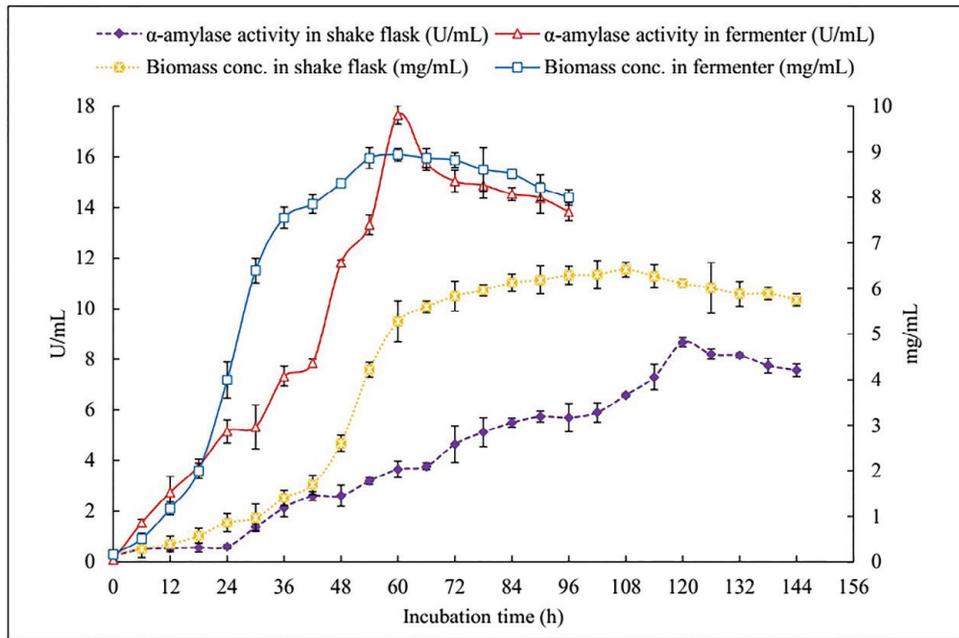
Figure 4. *Fermentation profile of* B. licheniformis-*AZ2.* α-amylase *produced under optimum fermentation condition using a fermenter and shake flasks. The temperature of fermenter was controlled at 37°C, pH 9.0, aeration rate of 1 vvm and agitation speed of 100 rpm. Shaken cultures were incubated at 40°C, initial pH 9.0 on a rotary shaker at fixed agitation speed of 120 rpm.*
Mean ± S.E.M = Mean values ± Standard error of means of three replications.
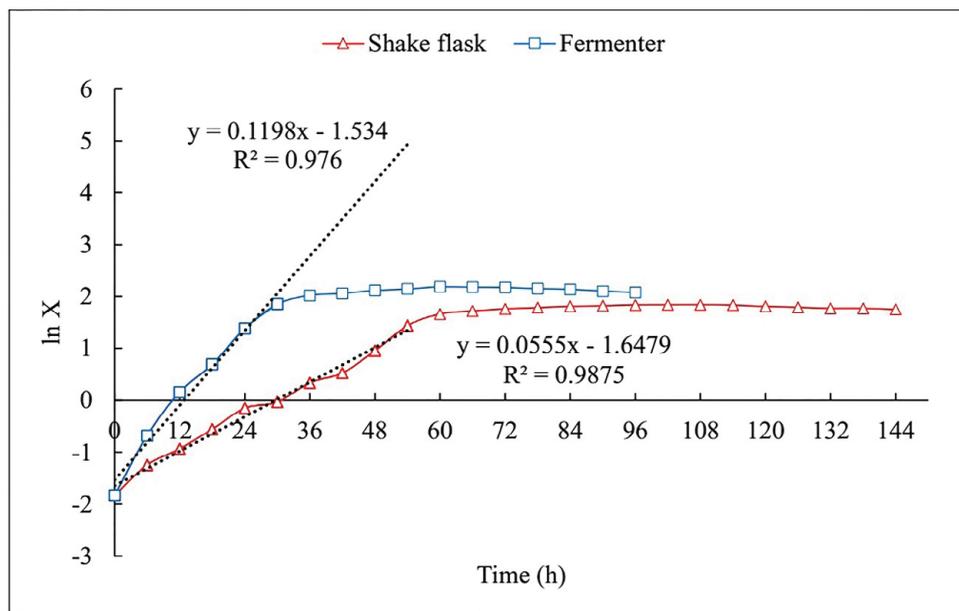
Figure 5. *Specific growth rates of* B. licheniformis-*AZ2 incubated for various fermentation time under optimum fermentation conditions using stirred fermenter and shake flask.*

menter cells grew exponentially after about 12 h of lag phase and reached to the stationary phase of growth by dry cell biomass of about 8.94 ± 0.25 mg/mL after 42 h. Peak α-amylase activity (17.66 ± 0.87 U/mL) in the stirred fermenter was observed in the mid stationary phase of growth after 60 h of fermentation time. This time was 60 hours

shorter than the corresponding batch culture in the shake flask. In the stirred fermenter, the fermentation was much faster than that in the shake flask, as indicated by biomass formation rates (Figure 4). This may be ascribed to improved aeration and mass transfer in the stirred fermenter. The natural log of biomass (ln X) against fermentation

Table 4. *Kinetic study of cell growth and α-amylase production by* B. licheniformis-*AZ2 under optimal fermentation conditions using stirred fermenter and shake flask*

Fermentation condition	Q_x^1 (g/L/h)	Q_p^2 (U/L/h)	$Y_{p/x}^3$ (U/g)	μ_{max}^4 (h^{-1})	q_p^5 (U/g/h)	t_d^6 (h)
Stirred Fermenter	0.40[a]	723.1[a]	45.17[a]	0.120[a]	5.41[a]	5.78[b]
Shake flask	0.27[b]	228.6[b]	13.64[b]	0.055[b]	0.76[b]	12.48[a]

Kinetic parameters: [1]Q_x = g cell mass formation/L/h, [2]Q_p = Enzyme produced/L/h, [3]$Y_{p/x}$ = Enzyme produced/g cell mass formation, [4]μ_{max} (h^{-1}) = Specific growth rate, [5]q_p = Product formation specific rate U/g/h, [6]t_d = Generation time (h).
Means with different letters indicate significant differences between treatments (*P* < 0.05, Duncan's multiple range test).

time was plotted for both shake flask and the stirred fermenter (Figure 5). In the stirred fermenter, no significant lag observed in the growth, most probably due to the optimal aeration and agitation. In the stirred fermenter, during the first 30 h, the specific growth rate was 0.1198 h^{-1}, and it gradually decreased in the next 6 h. This indicates that the true logarithmic growth phase lasted for the first 30 h. However, in shake flask, logarithmic growth was observed for the first 60 h, during which the specific growth rate remained at 0.0555 h^{-1}, afterwards, it gradually decreased, showing termination of the exponential growth phase. The generation times (t_d) during the exponential phases were calculated to be 5.78 h and 12.48 h in the stirred fermenter and shake flask, respectively.

DISCUSSION

The genus *Bacillus* has the capacity to produce a wide variety of extracellular enzymes with broad ranges of application, among them amylases particularly have considerable industrial importance *(31)*. Up scaling the fermentation processes from laboratory to commercial scales is always a challenging problem, because it is difficult to assess the impact of factors influencing the scale-up bioprocess during the fermentation *(32)*. It is well known that production of extracellular industrial enzymes by microorganisms in fermenters is greatly affected by media components and different physical factors *(33)*. Therefore, studying the effect of operational conditions such as incubation temperature, agitation speed and aeration rate on enzyme production might provide more reliable and comprehensive understanding about the enzyme synthesis patterns, other environmental requirements, influential parameters, and information about process scale-up *(34)*.

As synthesis of α-amylase correlated with bacterial growth, an improvement on bacterial growth environment would enhance α-amylase synthesis. It has long been established that temperature plays an important role in growth of bacteria and activity of their enzymes in fermentation medium *(35)*. In this study, α-amylase pro-

duction and growth of *B. licheniformis*-AZ2 were found to be optimal at 37°C. Similar results for growth and α-amylase production were reported for *B. licheniformis* ATCC 6346 *(17)* and *B. amyloliquefaciens* EMS-6 *(36)*. However, temperature optima beyond 37°C were also reported in literatures for *B. licheniformis* BT5.9 *(37)* and *B. licheniformis* *(38)*.

On the other hand, it has been demonstrated by Mamo and Gessesse *(39)* increasing the incubation temperature in *B. stearothermophilus* cultures would be accompanied by the decreased level of cell-free amylases and concomitantly a rise in the amount of cell-bound amylases so that total (cell-free and cell-bound) amylase activity at different temperature values remains almost stable. It has been suggested that such differences in the level of amylase activity may not be due to the direct effect of temperature on bacterial growth. Therefore, the other possible explanation could be that the level of cell-free amylase released into the fermentation medium might be indirectly affected by temperature. In bacterial cells, releasing extracellular enzymes into fermentation medium is strictly controlled by several mechanisms. One of these mechanisms which can be induced by temperature is the changes made in the nature of cell envelope (cell membrane and cell wall) *(26, 39, 40)*.

In *Bacilli*, it is also believed that the S-layer (surface protein layer) is affected by changes in oxygen levels in fermentation medium, which is associated with control of the extracellular enzymes secretion *(41–43)*. Thus, temperature through changing the level of dissolved oxygen in fermentation medium could indirectly affect the conformation of cell wall and S-layer, which thereupon leads to different temperature optima for growth and enzyme production *(39)*. Accordingly, releasing of α-amylase by *B. licheniformis*-AZ2 cells might have reduced at elevated temperatures (40°C, 45°C and 50°C) due to the poor solubility of oxygen and high rate of oxygen consumption (Figure 1).

Unlike most other *Bacilli* which are typically aerobic, *B. licheniformis* is known to be a facultative anaerobic which may afford it to grow in more ecological niches *(44)*. However, the production of α-amylase by *B. licheniformis* is generally accepted as an aerobic process *(26)*. Therefore, in order to fulfil the requirements of microorganism for high oxygen needs fermentation medium must be continuously supplied with gaseous oxygen. Otherwise, the oxygen transfer can become a major limiting factor for cell growth and metabolism *(45)*.

The cell biomass values also varied with changes in aeration rates and maximum cell biomass and α-amylase production appeared at optimal value of 1 vvm (Figure 2). This indicates that increase in aeration rates would yield a higher cell biomass. However, a decrease in cell biomass and α-amylase production was noted at ≥ 1.5 vvm aeration rates that might ascribe to inappropriate transfer rate of oxygen in the fermentation medium. Re-

cently a phenomenon called "impeller flooding" has been reported in some studies, in which the air stream up in the bioreactor increased along the stirrer shaft when higher aeration flows was accompanied by lower agitation speeds. In this case, a column of air surrounds the impeller, and there will no longer be a proper contact with the liquid in the vessel, which results in reduced air dispersion, poor mixing and diminished oxygen transfer rates. This phenomenon has been recommended to be avoided by finding an appropriate combination of aeration rate and agitation speed together *(46)*.

The optimal agitation speed for α-amylase production was determined to be in the range of 100-150 rpm, while increasing in agitation speed beyond 150 rpm significantly reduced the cell growth and enzyme production yield followed by foam formation in fermenter (Figure 3). It has been demonstrated that exerting higher agitation speeds using mechanical stirrers damage microbial cells through imposing high sheer stress, on the contrary, lower agitation speeds could cause improper mixing of nutrients which ultimately affect α-amylase yield in both cases *(47)*. All these efforts indicate that there should be a balance between aeration rate and agitation speed for the optimum yield of cell biomass and α-amylase production.

As it mentioned by Hessleink *(48)*, the catalytic activity of an organism will not be fully accomplished unless adequate levels of oxygen are constantly supplied in close proximity of the cells through aeration and agitation. Adversely, higher agitation speeds by induction of shearing forces and vortex formation in fermentation broth could also cause an impaired mass transfer rate and therefore reduced enzyme secretion. Current study also pointed out that higher agitation speeds can cause a drastic reduction in extracellular enzyme production by *B. licheniformis*-AZ2 (Figure 3).

As it can be deduced from Figure 4, the lag phase was significantly shortened after scaling up the fermentation process from shake flask to stirred fermenter in batch mode. In shaken cultures, the lag phase and acceleration of cell growth was elongated by approximately 42 h. While in stirred fermenter this time was much faster and after taking only about 16 h of incubation cells entered to logarithmic phase, which resulted in a shorter incubation time in stirred fermenter condition. The longer fermentation process in shake flask cultures was probably due to the limited oxygen uptake and gas exchange, because shake flasks are usually agitated at a fixed speed and most cultures are doomed to failure when the available ambient oxygen is depleted from the surroundings. Other limiting factors in batch shaken cultures could be lack of pH control and imprecise control of temperature *(49)*.

In general, the prolonged lag phase observed in this study in comparison of other studies with simple sugars as energy source might be due to the type of carbon source. It has been proven that the composition of agri-

cultural residues used in fermentation medium by affecting the length of the lag phase and primary metabolism could alter the time needed by microorganism for enzyme synthesis. Madar et al. *(50)* also stated that there is a programmed evolutionary gene expression during the lag phase for synthesis of crucial enzymes based on type of available carbon sources in fermentation medium, which helps microorganism with the best exploitation of biomass once exits from the lag phase. Therefore, simple carbon sources compel the organism to rapidly enter to long log-phase, leading to the formation of more dry cell biomass, while more complex carbon sources like agro-wastes cause a delay in the commencement of log-phase and increase in generation time.

The bacterial cells subsequently entered in exponential phase of growth with generation times of 12.48 h and 5.78 h in stirred fermenter and shake flask cultures, respectively. During the exponential phase of growth α-amylase production increased with increase in cell biomass which found to be growth-associated. There are some contradictory reports in literatures regarding the phase of growth in which *Bacillus* species show their maximal α-amylase production. As some reports have suggested that the highest enzyme activity is obtained in the exponential phase of growth, whereas some other indicated that it occurs at the mid-stationary phase (51). In the present study the highest levels of α-amylase production by *B. licheniformis*-AZ2 in both the shake flask and the stirred fermenter were observed at the stationary phase of bacterial growth (Figure 4).

The constant cell biomass yield of bacteria during the post-logarithmic (deceleration) and stationary phases suggests that substrate has been efficiently consumed by microorganism until stationary phase. This fact implies that enzyme production is not absolutely growth dependent and forceful induction of α-amylase may not occur unless the stationary phase of growth has been reached and readily available carbon sources were depleted from the fermentation medium *(52)*. Ultimately, after about 72 h and 114 h of process time in stirred fermenter and shake flask conditions, cell death dominated, which directly corresponds to secondary nutrient depletion and accumulation of toxic metabolite products *(53)*.

CONCLUSION

In our previous study, among the strains isolated from Qinarje Hot Spring *B. licheniformis*-AZ2 has been found as an appropriate strain for α-amylase production. We have also conducted optimization experiments in order to enhance α-amylase production in shake flask level and ultimately a low-cost fermentation medium containing rice husk was developed. In this study we have attempted to scale up enzyme production from 100 mL in shake flask to 1 L under stirred fermenter condition. Finding of the present study indicates a 2.1-fold increase in α-amylase

production under stirred fermenter condition with optimized agitation speed of 100 rpm and 1 vvm aeration rate at 37°C in a 60-hour-shorter process time compare to the shake flask experiments. Kinetic fermentation parameters also confirm the superiority of fermenter cultures. To be commercialized further studies are needed for scaling up in pilot-scale before testing larger samples in production scale. The enzyme production will have to be carried out in fed batch fermentation system in future.

Conflicts of Interest: *The authors declare no conflict of interest.*

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