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Production of cyclodextrin glucanotransferase from alkalophilic *Bacillus* sp. TS1-1: Optimization of carbon and nitrogen concentration in the feed medium using central composite design

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Abstract

Optimisation of nutrient feeding was developed to overcome the limitation in batch fermentation and to increase the CGTase production from *Bacillus* sp. TS1-1 in fed batch fermentation. Optimisation of the C/N ratio in the feed stream was conducted in a 5 l fermenter, where feeding was initiated at constant rate of 0.02 h^{-1}. In our initial screening process, the addition of nitrogen source boosted the growth of the microbes, but on the other hand reduced the CGTase production. The amount of tapioca starch and yeast extract was optimised in order to obtain a sufficient growth and thus, increased the CGTase production. Results were analysed using three-dimensional response surface plot, and the optimised values of carbon and nitrogen concentration of 3.30% (w/v) and 0.13% (w/v) were obtained, respectively. CGTase activity increased up to 80.12 U/ml, which is 13.94% higher as compared to batch fermentation (70.32 U/ml). This also led to 14.54% increment of CGTase production in fed batch culture as compared to the production before the optimisation. The CGTase activity obtained was close to the predicted value, which is 78.05 U/ml.

Keywords: Cyclodextrin glucanotransferase; C/N ratio; Fed batch; Central composite design

1. Introduction

Cyclodextrin glucanotransferase (EC 2.4.1.19) is an extracellular enzyme, a member of the amylolytic glucosylase family. CGTase differs from its family members, possesses a capability to catalyse multiple reactions [1]. It has both strong hydrolytic and synthetic capabilities as well as having multiple product specificity [2]. The ability of CGTase to convert starch into favoured industrial substance called cyclodextrin through cyclization process is of great interest to researchers [2]. Cyclodextrins are able to form an inclusion complex with various kinds of organic compounds inside the cavity of the ring structure [3].

Most production of the CGTase were carried out as batch processes [4,5]. However, CGTase production in batch processes show many limitations caused by substrates suppression, catabolite repression and limiting of some essential nutrients. Therefore, applying fed batch fermentation was constructive to overcome all the limitations in batch fermentation. Unfortunately, limited information was obtained regarding CGTase production using fed batch culture [6–8]. The main purpose of employing fed batch culture was to remove the repressive effects of rapidly utilized carbon sources, to reduce the viscosity of the medium, to reduce the effect of toxic medium constituents or simply to extend the product formation stage of the process for as long as possible. Besides CGTase, fed batch method had been proven to increase the production of proteases by *Bacillus sphaericus* [9] and production of enzyme β-1,4-endoglucanase from recombinant *Bacillus subtilis* DN18859 (pCH7) [10].

Media optimisation for feed stream using statistical experimental design for production of novel CGTase by fed batch fermentation had not been reported before. On the other hand, the optimising of media formulation in batch fermentation was read-

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ily available. Optimisation of either biomass growth, enzymes, certain extracellular proteins and bioactive metabolites in batch fermentation has been cited by many authors such as Lee and Chen [11], Dey et al. [12], Liu et al. [13], Khairizal et al. [14], Chen et al. [15], Li et al. [16] and Roshanida et al. [17].

Only few reports on the effect of C/N ratio on CGTase production by batch fermentation were available [4]. In the present study, optimisation of C/N ratio in the feed stream was conducted using central composite design in order to enhance the production of CGTase from *Bacillus* sp. TS1-1 by fed batch fermentation.

2. Materials and methods

2.1. Preparation of bacteria inoculum

Bacillus sp. TS1-1 is provided by our own laboratory [14]. The bacteria was isolated from the soil and grown in an optimised medium with the composition of 2% (w/v) sago starch (food grade), 1% (w/v) yeast extract, 0.1% (w/v) K_2HPO_4 and 0.02% (w/v) $MgSO_4{\cdot}7H_2O$ [14]. The medium was added with 10% (w/v) Na_2CO_3 stock solution, separately depending on the pH of the medium. *Bacillus* sp. TS1-1 was cultured in 250 ml conical flasks and incubated in an orbital shaker at of 37 °C and 200 rpm for 18 h. The cells were then centrifuged at 5000 rpm for 5 min, washed once with normal saline solution 0.85% (w/v) NaCl, to give an optical density (OD) reading of 0.5 at 600 nm.

2.2. CGTase activity assay

CGTase assay was carried out according to the method of Kaneko et al. [18]. The reaction mixtures containing 40 mg of soluble starch in 1.0 ml of 0.1 M sodium phosphate buffer (pH 6.0) and 0.5 ml supernatant was incubated at 60 °C for 10 min. The reaction was stopped by adding 3.5 ml of 30 mM NaOH, followed by 0.5 ml of 0.02% (w/v) phenolphthalein in 5 mM Na_2CO_3 solution, and left for 15 min at room temperature. The colour intensity of the samples was measured at 550 nm. A blank solution (fresh medium) was prepared for each batch of assay. One unit of enzyme activity was defined as the amount of enzyme that forms 1 μmol of β-CD/min.

2.3. Biomass determination

Two methods were carried out for the determination of biomass concentration, with regards to presence or absence of starch in the culture broth [19]. If the culture contains traces of starch, 0.1 ml enzyme α-amylase (Novo Nordisk) was added to 1 ml of culture to hydrolyse the residual starch to soluble sugars. The mixture was incubated at 100 °C for 20 min and centrifuged at 3000 rpm for 30 min. The cells were filtered onto pre-weighed 0.2 μm cellulose nitrate filter (Whatman), washed twice with distilled water and dried in an oven at 95 °C for 24 h. Fresh medium was treated similarly and used as blank. If starch was totally depleted from the sample: 1 ml of culture was centrifuged at 5000 rpm for 3 min. The cell pellet was washed twice with saline solution and dried in a vacuum dryer, followed by drying in the oven at 80 °C.

2.4. Starch concentration

Determination of starch concentration was carried out according to the method of Kitahata et al. [20] 1.0 ml of supernatant was mixed with 4 ml of 0.01 M iodine in 0.25 M potassium iodide (KI) and diluted with 15 ml of distilled water. The colour intensity was measured at 465 nm against blank of distilled water treated in the same manner.

2.5. Protein content

Protein assay was carried out according to Lowry [21] 0.2 ml of each standard or crude enzyme was mixed with 1.0 ml of Lowry reagent and left at room temperature for 10 min. At the end of the incubation period, 0.1 ml of the diluted reagent Folin–Ciocalteu (1 distilled water:1 folin solution) was immediately mixed to the reaction samples, and left at room temperature for another 30 min. The absorbancy of the sample was measured at 750 nm.

2.6. Calculation of C/N ratio

CN ratio was calculated on molar basis. One gram of starch/l is converted to 1.1 g of glucose/l. The formula used for the conversion of mass concentration of starch to (g/l) to mM concentration of carbon was [starch (g/l) × 36.7 = mM carbon]. The total nitrogen content in the yeast extract used is 11.4%. The conversion of yeast extract mass concentration (g/l) to mM concentration of nitrogen is [yeast extract (g/l) × 11.4 = mM nitrogen].

2.7. Fermentor set-up

All fermentations processes were carried out in a 5 l borosilicate glass fermenter (with working volume of 4 l, Biostat® B, Germany), with an internal concave bottom of height and diameter ratio of about 2:1 (dimension $W \times H \times D = 400$ mm × 685 mm × 330 mm). The fermenter was equipped with a peristaltic pump, temperature, pH and dissolved oxygen (DO) controllers. A polagraphic dissolved oxygen probe (Ingold, Switzerland) was used to measure the DO levels, and a glass pH electrode (Ingold) was used to measure the culture pH. The fermenter was fitted with three six bladed Rushton turbine impellers (diameter, $d = 40$ mm) on the agitator shaft and the impeller speed was fixed at 200 rpm.

The initial cultivation medium that contained (w/v): 2% starch, 1% yeast extract, 0.1% K_2HPO_4 and 0.02% $MgSO_4{\cdot}7H_2O$ was added with 10% of sterilised Na_2CO_3 separately to give a final pH of 10.32. The media was sterilized at 121 °C for 15 min and sterilization of the fermenter was carried out by autoclaving for 20 min. About 10% (v/v) of bacterial inoculums were inoculated into the cultivation medium for both batch and fed batch fermentation. Cultivation was carried out at 29.6 °C for 48 h at 200 rpm, with dissolved oxygen levels (DO) con-

Table 1
Actual and coded values of the design variables for the optimisation process using the fed batch process

Factors	Low level star point (−1.682)	Low level factorial (−1)	Centre point (0)	High level factorial (+1)	High level star point (1.682)
X_1: tapioca starch (%, w/v)	0.50	1.50	3.00	4.50	5.50
X_2: yeast extract (%, w/v)	0.003	0.13	0.31	0.50	0.63

The optimisation was performed to the total sets of 17 experiments, consisting eight factorial points, four axial (star) points and five centre points using response surface methodology.

trolled at 20–50% saturation. Batch operation in a 5 l fermenter was carried out as a control with initial volume of 4 l cultivation medium. All experiments were repeated for three times to obtain precise data.

2.8. Fed batch operation

Fed batch fermentation was carried out using the same bioreactor and conditions as mentioned in batch fermentation, with initial volume reduced to $V_0 = 2$ l. The cells were initially grown in batch mode, and feeding was initiated when residual starch was nil and biomass reached its maximum ($X = X_{max}$) within 16–18 h of incubation. The medium was fed at constant rate of 0.02 h^{-1} (based on previous study) from the beginning of feeding to the end of cultivation. The DO level was kept at 20–50% air saturation by adjusting the aeration rate. pH of the culture was not controlled since based on the previous study showed that maximum CGTase activity was obtained using natural pH control strategy [22].

2.9. Experimental design and optimisation

The experimental design was carried out using Design Expert Software (Stat-Ease Inc., Statistics made easy, Minneapolis, MN, USA, Version 6.0.4) [23]. Central composite design (CCD) was used to identify the optimum carbon and nitrogen concentrations in the feed medium, in order to obtain maximum CGTase production (Y_1), besides evaluating the interaction between C/N ratio and the biomass production (Y_2) as the second responses. The design consisted of eight factorial points, four axial (star) points and five centre points, giving total sets of 17 experiments (Table 1). The collection of experiments provides an effective means for optimisation through these process variables. Besides, the design permits the estimation of all main and interaction effects. On the other hand, the purpose of the centre points was to estimate the pure error and curvature. The quadratic model for predicting the optimal point was expressed according to the following equation:

$$Y = b_0 + \sum b_i X_i + \sum b_{ii} X_i^2 + \sum b_{ij} X_i X_j \quad (1)$$

where Y is response variables; b the regression coefficients of the model; X represents the coded levels of the independent variables. The regression equation above was optimised for optimal values also using *Design Expert* software. The statistical significance of the second-order model equation was determined by F-value and the proportional of variance explained by the model obtained was given by the multiple coefficient of determination, R^2. The experiment was conducted in a randomized order to avoid systematic bias. Interpretation of results was analysed by

Table 2
Experimental layout of the design variables

Run	Factor 1 X_1: carbon		Factor 2 X_2: nitrogen		Response (Y)	
	Actual value	Coded value	Actual value	Coded value	CGTase activity (U/ml) (Y_1)	Biomass (g/l) (Y_2)
1	1.5	−1	0.13	−1	61.20 ± 1.50	10.6 ± 0.31
2	1.5	−1	0.13	−1	62.75 ± 0.90	10.2 ± 0.28
3	4.5	1	0.13	−1	72.29 ± 1.71	13.6 ± 0.27
4	4.5	1	0.13	−1	73.44 ± 1.23	13.9 ± 0.31
5	1.5	−1	0.5	1	62.33 ± 0.67	11.4 ± 0.16
6	1.5	−1	0.5	1	60.81 ± 0.35	11.2 ± 0.14
7	4.5	1	0.5	1	70.86 ± 1.53	17.4 ± 0.52
8	4.5	1	0.5	1	69.41 ± 1.02	17.6 ± 0.24
9	0.5	−1.682	0.31	0	50.12 ± 2.32	10.2 ± 0.37
10	5.5	1.682	0.31	0	63.03 ± 1.82	15.8 ± 0.16
11	3	0	0.003	−1.682	80.12 ± 2.29	11.7 ± 0.12
12	3	0	0.63	1.682	72.88 ± 2.11	16.3 ± 0.40
13	3	0	0.31	0	77.26 ± 2.49	15.5 ± 0.84
14	3	0	0.31	0	76.80 ± 1.24	15.6 ± 0.53
15	3	0	0.31	0	78.34 ± 2.87	14.7 ± 1.15
16	3	0	0.31	0	78.41 ± 1.53	16.1 ± 1.05
17	3	0	0.31	0	77.62 ± 1.19	15.1 ± 0.67

The maximum CGTase activities and biomass production of each run were chosen as responses.

analysis of variance (ANOVA) as appropriate to the experimental design used. The results are shown in Table 2.

3. Results and discussion

3.1. Growth and CGTase profile from batch fermentation

Prior to fed batch study (optimisation of C/N ratio), batch fermentation was carried out in order to study the CGTase production and the growth profile of *Bacillus* sp. TS1-1. Batch fermentation was carried out using 2% (w/v) gelatinised sago starch as sole carbon source. The biomass of *Bacillus* sp. TS1-1 increased exponentially and reached its maximum value after 20 h of incubation (Fig. 1). The CGTase production reduced when growth of the microbes entered the stationary phase. Maximum CGTase activity of 70.32 U/ml with specific activity of 0.198 U/μg was attained after 28 h, with maximum biomass (X_{max}) of 10.1 g/l. The specific growth rate (μ) of *Bacillus* sp. TSI-1 was 0.169 h^{-1}, with doubling time (τ_d) of 4.1 h. Production of CGTase by *Bacillus* sp. TS1-1 was a mixed growth associated, and maximum CGTase activity was observed during the stationary phase.

3.2. Screening of fed batch modes and types of carbon and nitrogen sources

In our initial study, few parameters influencing the CGTase production such as the feeding mode, types of carbon and nitrogen sources were screened using conventional method (data not shown). From our screening process, constant feed rate fed batch at rate of 0.02 h^{-1}, tapioca starch of 2% (w/v) and yeast extract of 0.5% (w/v) were selected as the best parameter enhancing the CGTase production and the biomass concentration in fed batch fermentation. The highest increment of CGTase activity and specific activity were obtained using constant rate feeding mode and tapioca starch. Feeding at higher and lower dilution rate resulted in lower CGTase production. Whilst, the addition of 0.5% (w/v) of nitrogen source in the feeding medium resulted in higher biomass concentration, on the other hand it reduced the CGTase activity as compared to the batch fermentation. Therefore, it is very important to find out an appropriate amount of substance to be fed to the culture that would increase the growth of the microbes and thus, increase the product yield.

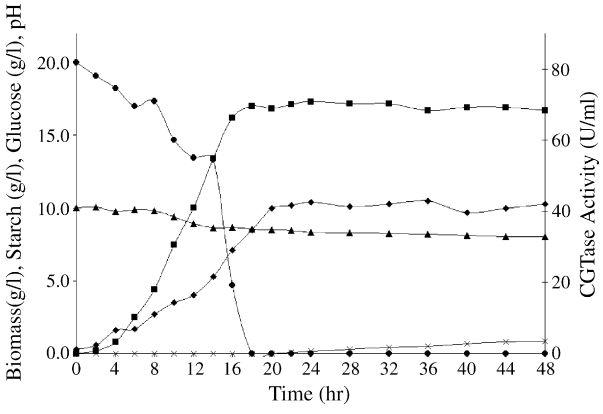

Fig. 1. Growth, starch consumption, pH, glucose concentration and CGTase production profiles by *Bacillus* sp. TS1-1 grown at 29.6 °C, 200 rpm in 5 l fermenter with DO control at 20–50% air saturation (batch cultivation). Symbol represent: (–■–) CGTase activity; (–♦ –) biomass; (–▲–) pH; (–×–) glucose concentration; (–●–) starch concentration.

Table 3
Experimental and predicted values of CGTase activity (U/ml)

Standard order	Actual value ($\hat{Y}$)	Predicted value ($\hat{Y}$)	Residual ($Y - \hat{Y}$)
1	61.20	64.45	−3.25
2	62.75	64.45	−1.70
3	72.29	74.49	−2.20
4	73.44	74.49	−1.05
5	62.33	62.91	−0.58
6	60.81	62.91	−2.10
7	70.86	70.62	0.24
8	69.40	70.62	−1.22
9	50.12	47.01	3.11
10	63.03	61.94	1.09
11	80.12	76.68	3.44
12	72.88	72.13	0.75
13	77.26	76.99	0.27
14	76.80	76.99	−0.19
15	78.34	76.99	1.35
16	78.41	76.99	1.42
17	77.62	76.99	0.63

Coefficient of correlation, $R = 0.9322$; coefficient of determination, $R^2 = 0.9534$. The correlation coefficient, $R = 0.9322$ justifies a good correlation between the independent variables.

3.3. Experimental design—effect of CN ratio in feeding medium on CGTase production in fed batch culture

Before the optimisation process, fed batch study was started with feeding of 2% (w/v) tapioca starch and 0.5% (w/v) yeast extract. The production of CGTase and the specific activity were slightly reduced to 69.77 ± 1.17 U/ml and 0.164 U/μg protein, respectively (data not shown) as compared to the batch fermentation. On the other hand, the biomass production was increased up to 69.3%. It is necessary to carried out the optimisation process in order to find out the appropriate concentration of these substrates, hence increased the CGTase production.

The carbon (X_1) and nitrogen concentration (X_2) were optimised using central composite design. Tables 2 and 3 summarised the central composite design matrices and the experimental and predicted response for each individual experiment, respectively. The multiple regression equation for the CGTase activity (Y_1) in fed batch cultivation by *Bacillus* sp. TS1-1 with process conditions of carbon (X_1) and nitrogen concentration (X_2) as the main variables is shown below (Table 4):

$$Y_1\ \text{(U/ml)} = 33.93 + 24.93X_1 + 15.83X_2 - 3.45X_1^2 - 26.73X_2^2 - 2.10\ X_1X_2 \quad (2)$$

Based on the experimental results for CGTase production, a quadratic model for CGTase activity with functions of carbon and nitrogen concentrations was derived. The quadratic model generated from the software consisted of one offset, two linear, two quadratic and one interaction terms. The model adequacy

Table 4
Regression analysis (ANOVA) for the production of CGTase by *Bacillus* sp. TS1-1

Source	Sum of squares	d.f.	Mean square	*F* value	Prob > *F*[a]
Model	1089.70	5	217.94	45.03	<0.0001
A: tapioca starch	269.01	1	269.01	55.58	<0.0001
B: yeast extract	24.94	1	24.94	5.15	0.0443
A^2	782.48	1	782.48	161.66	<0.0001
B^2	10.34	1	10.34	2.14	0.1719
AB	2.71	1	2.71	0.56	0.4696
Residual	53.24	11	4.84		
Lack of fit	47.24	3	15.75		
Pure error	6.01	8	0.75		
Correlation total	1142.94	16			

d.f.: degree of freedom.
[a] Values of *p*-values less than 0.0500 indicate model terms are significant.

was verified using Fisher test (*F* test) and the determination coefficient, R^2. The analysis of variance (ANOVA) for the selected quadratic model of the design is tabulated in Table 5. From the ANOVA analysis, the confidence level was greater than 99.91% ($p > F = 0.0001$) for CGTase production with *F*-value of the model was 45.03, with probability of <0.0001. The high *F*-value and a very low probability ($p > F = 0.0001$) indicated that the present model was showing good agreement between predicted and experimental results. Therefore, the obtained mathematical model was adequate.

The fitness of the model was expressed by the coefficient of determination R^2, which was calculated to be 0.9534, indicating that 95.34% of the variability in the response could be explained by the model and only 4.66% of the total variation were not explained by the model. A higher value of coefficient of correlation (*R*) justified an excellent correlation between the independent variables. In other words, if the *R*-value ≥ 0.9, the correlation between the experimental and predicted values was significant. The *R*-value (0.9322) indicated a good agreement existed between the experimental and predicted values of CGTase activity.

Table 5
Comparative performance between batch and after optimisation (fed batch)

Properties	Batch	Optimised condition (fed batch)
Initial pH	10.32	
Temperature (°C)	29.6	
Inoculum size (%, v/v)	10	
Agitation rate (rpm)	200[a]	
Specific growth, μ_{max} (h^{-1})	0.169	0.173
Doubling time, τ_d (h)	4.10	4.03
Maximum biomass, X_{max} (g/l)	10.1 ± 0.15[b]	13.2 ± 0.23[b]
Maximum CGTase activity (U/ml)	70.32 ± 1.24[c]	80.12 ± 1.43[c]
Maximum specific activity (U/μg)	0.198[b]	0.220[c]
Exponential phase (h)	22	40
Time to reach maximum CGTase activity	After 22 h	After 38 h

[a] Typical agitation rate depending on culturing methods.
[b] The data were observed at 48 h of incubation.
[c] The data were observed at maximum CGTase activity.

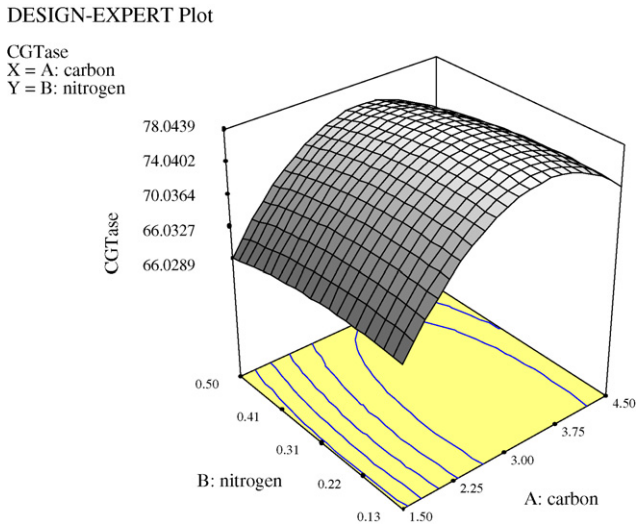

Fig. 2. The three-dimensional presentation of the response surface for the CGTase activity of *Bacillus* sp. TS1-1. The model had predicted the optimum CGTase activity to be 78.05 U/ml with the tapioca and yeast extract of 3.3% (w/v) and 0.13% (w/v), respectively.

Fig. 2 represents the model (Eq. (1)) obtained for the CGTase production. From the response surface plot (Fig. 2), the production of CGTase was increased by simply increasing the tapioca starch up to certain limit, whereas the yeast extract concentration was reduced to the minimum. It is possible to conclude that the production of CGTase by fed batch fermentation system requires minimum concentration of nitrogen source (yeast extract). The yeast extract concentration must be kept as low as possible to avoid repression caused by excessive nitrogen source, thus leads to the reduction of CGTase production.

Increased of tapioca starch concentration resulted in the increment of both CGTase activity and biomass production. However, when the concentration of starch in the feed stream exceed beyond 3% (w/v), the CGTase activity dropped significantly. High concentration of substrate will reduce the CGTase production due to the increment of viscosities of the culture that lead to poor oxygen uptake [24]. Besides, increased substrate also caused the production of high concentration glucose or other low metabolisable sugars (Table 6). A study carried out by Shene et al. indicated that the increment of residual glucose concentration in culture broth even at the lowest value would repressed the production of recombinant β-1,4-endoglucanase [10]. The same repression also occurred in the production of glucoamylase by *B. subtilis* strain 11089 [25] and in the production of alkaline protease from *B. sphaericus* [9]. The repression in protease production occurred when the culture was fed with high concentrations of glucose and yeast extract.

However, in this study it was observed that the increment of biomass beyond certain value (>14 g/l) reduced the CGTase production. In the present study, low CGTase productions are correlated to high biomass concentrations. At high biomass concentration, the culture fluid became very viscous, which lead to reduction of dissolved oxygen content (DO) to less than 5% saturation. This could be the reason for the poor enzyme production. The effect of DO concentration on the CGTase pro-

Table 6
Effect of carbon and nitrogen concentration in the feeding medium during fed batch fermentation

Tapioca starch % (w/v)	Yeast extract % (w/v)	C/N ratio	CGTase activity[a] (U/ml)	Biomass (g/l)[b]	Glucose (μg/ml)
1.5	0.13	37.05	62.75 ± 0.90	10.2 ± 0.28	0.3322 ± 0.014
1.5	0.50		60.81 ± 0.35	11.3 ± 0.14	0.5617 ± 0.035
3.0	0.003	3211.13	80.21 ± 2.29	11.7 ± 0.12	1.0519 ± 0.032
3.0	0.30	31.08	76.80 ± 1.24	15.6 ± 0.53	1.4810 ± 0.047
3.0	0.63	15.29	72.88 ± 2.11	16.3 ± 0.40	2.0224 ± 0.028[c]
4.5	0.13	111.15	72.29 ± 1.71	13.6 ± 0.27	2.5240 ± 0.022
4.5	0.50	28.90	69.41 ± 1.02	17.6 ± 0.24	2.6533 ± 0.018

[a] Data taken at maximum CGTase activity.
[b] Data from taken at the end of fermentation process.
[c] Data taken at maximum glucose concentration during fed batch fermentation, standard deviation obtained from repetitions.

duction was studied using one-step increment in agitation rate. Fermentation with higher agitation rate (400 rpm) increased the DO level to 20–40% saturation, which lead to 15% increment in CGTase production (87.34 ± 2.13 U/ml) compared to production at 200 rpm (76.18 ± 1.24 U/ml). The same pattern was observed in the production of α-amylase from *Bacillus amyloliquefaciens*. The enzyme production increased up to 50% when dissolved oxygen in culture fluid increased from 10% to 60% saturation [26]. Medium with high starch concentration might as well increase the viscosity of the overall medium. A report on protease production showed that the enzyme activity and the overall yield were reduced when starch at high concentration was fed to the culture [27].

The high level of yeast extract concentration (low C/N ratio) allowed the strain to reproduced, thus enhancing the growth, making the enzyme synthesis less preferable. When C/N ratio was reduced from 3211.13 to 31.08, the CGTase activity was slightly reduced, up to 3.7%. However, the final biomass concentration (X_{max}) showed an increment up to 23.0% at lower C/N ratio. The interaction between carbon and nitrogen source on CGTase production are shown in Fig. 3. From Fig. 3, CGTase activity at 1.5% (w/v) of tapioca starch was higher with low level of nitrogen source, compared to high level of nitrogen source. These phenomena show that both carbon and nitrogen source are critical factors on CGTase production.

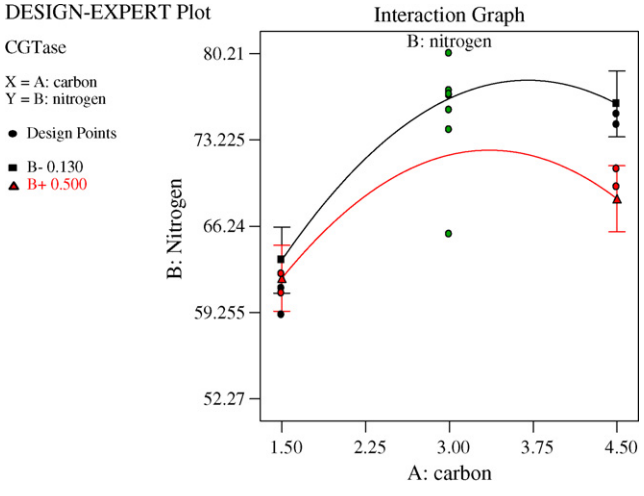

Fig. 3. Interaction graph of carbon and nitrogen concentration on CGTase activity from *Bacillus* sp. TS1-1 by fed batch fermentation.

The response surface plot (Fig. 2) depicts that the optimal carbon and nitrogen concentration for CGTase production were 3.30% (w/v) and 0.13% (w/v), respectively, giving the C/N ratio of 91.39. The model prediction for maximum CGTase activity at the optimum carbon and nitrogen concentration was 78.05 U/ml. To confirm the accuracy of the model prediction, an experiment was performed using the optimised condition obtained from the response surface plot. The maximum cell growth was obtained after 34 h, whereas the maximum CGTase activity of 80.12 ± 1.43 U/ml was attained after 36 h of fermentation. Nevertheless, the CGTase activity was almost stationary after 40 h of incubation. This phenomenon might be due to the presence of CD in the process medium, which suppressed the CGTase production. CGTase activity from *Bacillus* BE101 had been distorted by CDs, causing 50% reduction of CGTase production for every 6.0 g/l β-CD [28]. The specific activity obtained for optimised condition of 0.22 U/μg was slightly higher as compared to the batch fermentation (0.198 U/μg protein). An increment of CGTase activity by 13.94% was obtained using the optimised condition as compared to batch fermentation.

3.4. Effect of C/N ratio in feeding medium on biomass production in fed batch culture

The multiple regression equation for the final biomass production (Y_2) achieved during fed batch culture with various carbon and nitrogen concentration was as follows. The three-dimensional plot was shown in Fig. 4.

$$Y_2\ (\text{g/l}) = 5.63 + 3.19X_1 + 10.51X_2 - 0.43X_1^2 - 18.21X_2^2 + 2.57X_1X_2 \quad (3)$$

The adequacy of the model obtained for biomass production in fed batch culture was also verified using the F test. The F value obtained from this model is 38.34 (data not shown), which is higher than the tabulated F value, $F_{5,11,0.01} = 5.32$ (with significance level set at 1%). Thus, this also indicated that the model above could be used to predict precisely the biomass production in fed batch culture. The fitness of the model was expressed by the coefficient of determination, R^2 that was calculated to be 94.57%. The value of coefficient for both tapioca starch and yeast extract showed similar effect on the biomass production (the coefficient of +3.19 and +10.51, respectively). However, it can be clearly seen that yeast extract have the greater effect

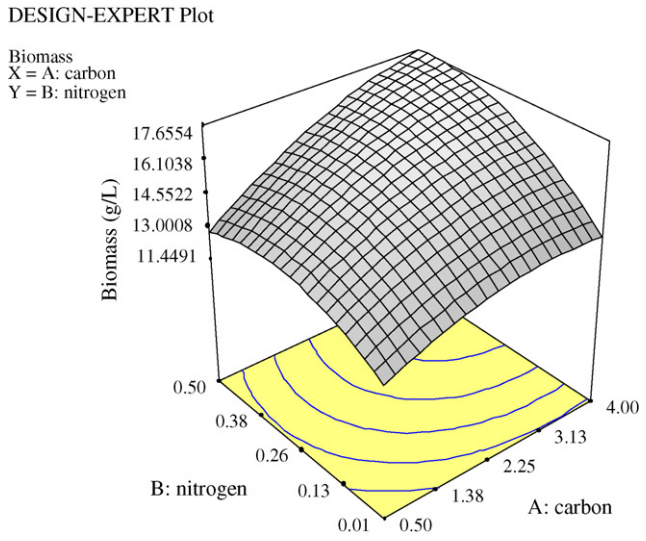

Fig. 4. The three-dimensional presentation of the response surface for the biomass production of *Bacillus* sp. TS1-1. The model showed that high carbon and nitrogen concentration gave maximum biomass production. The R^2 obtained for the model was 94.57%.

on the enhancement of the biomass production as compared to tapioca starch.

The biomass concentration of *Bacillus* sp. TS1-1 increased steadily with the increment of both tapioca and yeast extract concentrations in the feeding medium (Table 6). However, the increment was not in the exact proportion to the amount of nutrient fed to the systems. At 3% (w/v) of tapioca starch, with different C/N ratio, the amount of the maximum biomass and CGTase activity obtained were also varied. Higher C/N ratio gives rise to CGTase activity, where as the biomass production is slightly repressed. This could be due to the insufficient supply of nitrogen source.

However, when the C/N ratio was reduced (yeast extract concentration increased) the biomass production (for feeding with 4.5% (w/v) tapioca starch) was increased from 13.6 g/l (C/N: 31.08) to 17.4 g/l (C/N: 5.29). In contrast, the CGTase activity was reduced for 17.7%. This observation was in agreement with Youssef et al. and Auer Seviour [29,30]. The production of pullulan from *A. pullulans* by fed batch fermentation was reduced greatly when nitrogen source was fed in excess, and low CN ratio was preferable for the biomass production [30].

Feeding with 4.5% (w/v) of tapioca starch and 0.5% (w/v) yeast extract prolonged the exponential phase of growth to the end of fermentation period. Nevertheless, the profile of CGTase production did not show a proportional increment to the biomass production (data not shown). This might be due to some of CGTases that were produced during the active growth were retained and anchored to the membrane cells. According to Pocsi et al. [31], the retained CGTase would only be released to the medium when the growth entered the late exponential phase or during the cell lysis.

Feeding of high concentration of yeast extract increased the biomass production. On the other hand, the presence of excess nitrogen could have induced the hydrolysis of starch to produce high concentration of simple sugar (Table 6). Therefore, only a small amount of nitrogen was required to maintain the sustenance of the viable cell. High amount of yeast extract would totally defeat the objective to increase the CGTase production.

4. Conclusions

The optimum concentration of carbon and nitrogen sources of the optimisation process were observed to be 3.30% (w/v) and 0.13% (w/v), respectively, which lead to the highest CGTase activity of 80.12 ± 1.43 U/ml (results from three replications). This showed an increment of 14.54% to the CGTase production after the optimization of CN ratio in the feeding medium compared to the initial production, which only gave 69.77 U/ml.

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