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OPTIMAL CONDITIONS FOR β-MANNANASE PRODUCTION BY *BACILLUS CIRCULANS* NT 6.7

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ABSTRACT

Optimum medium for mannanase production is very important and producing medium has been shown effective for mannanase production from *Bacillus circulans* NT 6.7 but expensive. This work aim to find low cost medium and optimum condition for mannanase production from *Bacillus circulans* NT 6.7. 1.5% concentration of defatted copra meal as carbon source in shake flask resulted in highest growth and enzyme activities of 10.51±0.53 Log CFU/ml and 12.84±4.38 U/ml at 18 hours accordingly. Also, 2% defatted copra meal in fermenter produced highest growth of 7.94±0.41 Log CFU/ml and enzyme activities of 32.07±2.94 U/ml at 15 hours. Using taguchi design, optimal conditions for inoculum, agitation and aeration were 1%, 600 rpm and 0.75 vvm, respectively. Under this optimized condition, the mannanase experimental yield (29.57±4.81U/ml) closely matched the yield predicted by the statistical model (24.15U/ml) with $R^2 = 0.75$. Using mineral salts medium, slightly increased in mannanase product was observed compare to previously used producing medium. Also, the cost of using mineral salts medium (cost per liter) is 27 time lower than the cost of producing medium. Suggesting that, mineral salt medium provides an economically feasible medium for producing mannanase from *Bacillus circulans* NT 6.7.

Keywords: *Bacillus circulans* NT 6.7, optimal condition, mannanase production, defatted copra meal, mineral salt medium

INTRODUCTION

Copra meal or coconut meal is a by-product from coconut milk and coconut oil processing. After pressing or extracting the oil, the remaining residue is called copra meal. Copra meal structure is galactomannan that consists of beta-D-1, 4-mannopyranose backbone with branch points from their 6-positions linked to alpha-D-galactose **(Burhanudin and Dingle, 2006)**. **Lin and Chen) 2004(** reported the proximate analysis of copra meal and defatted copra meal. Copra meal and defatted copra meal had protein content of 4.7 and 19.2%)w/w(, carbohydrate content was 25.3 and 80.1%)w/w(, fat content of 72.1 and 0%)w/w(, ash content of 1.8 and 1.6%)w/w(and moisture content of 2.7 and 2.8%)w/w(, respectively. Copra meal residue after the coconut oil processing had fat content in ranges from 1% to 22% **Göhl (1982(**. Copra meal is good substrate for produce the mannanase because the copra meal contains 5-30% mannan of pure mannan and galactomannan. Residue cake from coconut frequently discharged as a by-product in the process of oil extraction from copra, which contains large amount of mannose in the form of copra mannan **)Hossain *et al.*, 1996(**.

Beta-mannanase)Endo-1, 4-β-mannanase, EC.3.2.1.78(is hydrolytic enzymes which catalyze randomly β-1,4 mannopyranosyl linkages within the backbones of mannan, glucomannan, galactomannan and galactoglucomannan **)Stoll *et al.*, 1999(**. This enzyme is produced by several living organisms and it has been widely used in the industrial application. *Bacillus circulans* NT 6.7 was isolated from soil of coconut industry from Nakornpathom province. Beta-mannanases from *Bacillus circulans* NT 6.7 has high enzyme activity for locust bean gum) LBG(, 0.306 unit/ ml with appropriate optimum temperature and pH for industrial applications **)Phothichito *et al.*, 2006(**. Defatted copra meal used for mannanase production in a producing medium in 5-l fermenter cultivation: 600 rpm, 0.75 vvm and 45 °C. *Bacillus circulans* NT 6.7 exhibited the highest cell growth and mannanase activity with 9.39 Log CFU/ml and 27.70 units/ml at 6 hours, respectively. **) Pangsri *et al.*, 2015(** . These results were similar to the results of **Phothichitto *et al.*) 2006(** , who reported the optimum pH and temperature of crude enzymes from *Bacillus circulans* NT 6.7 were pH 6.0 and 50 °C, respectively.

Beta-mannanases are useful in many fields including in oil drilling operations **) You *et al.*, 2016(** , bioconversion of biomass wastes to fermentable sugars **)Chandrakant and Bisaria, 1998(**, upgrading of animal feed stuff **)Ray *et al.*, 1982(**, and reducing the viscosity of coffee extracts and fruit juice clarification **)Chauhan *et al.*, 2014(**. They also have potential application in the production of mano-oligosaccharides that are selectively utilized by intestinal *Bifidobacterium spp*. and also as valuable food sweetener or additive **)Tomotari, 1990(**.

Media composition play important role in the growth and overall activities of microorganisms. Different ingredients of media such as carbon sources, nitrogen sources, minerals, metal ions, polyols and additives have different effects on growth and activity of microorganisms. Thus, these parameters needs to be optimized to obtain optimal growth condition for microorganism. Optimum condition for better production yields of desired enzyme at low cost is a significant parameter for making the production process industrially viable.

The aims of this study was to develop a low-cost medium and find the optimal condition for mannanase production by *Bacillus circulans* NT 6.7. Preliminary data from the study showed that mineral salts medium has a lower price than other medium. Hence, was used in the study to produce mannanase.

MATERIAL AND METHODS

Materials

The copra meal was dried at 60 ºC for 4 hours. After that, the copra meal were blended and milled by Hammer mill to obtain the product with the particle size of 0.5 mm. The defatted copra meal was prepared by oil extraction in Soxhlet apparatus for about 4-6 hours.

Development of culture medium

Bacillus circulans NT 6.7 was used in the enzyme production. The bacteria was kept -20 ºC in Nutrient broth)NB(containing 20%)w/v(glycerol solution, and propagated twice at 45 ºC before use. NB medium was used to cultivation of inoculum for produce enzyme. The mineral salts medium) MSM(for fermentation study was prepared by adding 1% defatted copra meal and producing enzyme medium)PM(adding 1% defatted copra meal used as control. The MSM was chosen to study the effects of carbon source using defatted copra meal at varying concentrations)0.5% , 1% , 1.5% and 2% (w/v)(. The best concentration for highest activity was used to study in a fermenter.

Bacillus circulans NT 6.7 was used in the fermentation experiments followed the condition for enzyme production by **Pangsri *et al.*) 2015(**. The nutrient broth medium was used for cultivation of inoculum for the fermentation in the fermenter. The MSM for fermentation study was prepared by adding)1%, 1.5%, 2% (w/v)(defatted copra meal, NH_4NO_3 0.03% , $MgSO_4.7H_2O$ 0.02 % $FeSO_4.7H_2O$ 0.001 % $CaCl_2.2H_2O$ 0.005 % K_2HPO_4 0.754 %, KH_2PO_4 0.232 %, adapted from **Mabrouk and Ahwany (2008**(and was autoclaved at 121°C for 30 min.

Determination of growth temperatures on mannanase production

Determination of growth temperatures

The effective isolate was cultivated in 5 ml of NB medium under aerobic conditions with shaking at 150 rpm for 24 hours at 45 ˚C. Following that, 3% culture broth was transferred into 300 ml of PM with shaking at 150 rpm in varying temperature of 30, 35, 37, 45 and 50˚C. Sample were collected at 0, 4, 8, 12, 16, 20 and 24 hours of incubation. Upon that, the culture media were diluted and spread-plated for cells counting, whiles the supernatant or filtrate culture was assayed for mannanase activity.

Determination of growth pH on mannanase production

The strain NT6. 7 was cultivated in 5 ml of NB medium producing enzyme medium under aerobic conditions with shaking at 150 rpm for 24 hours at 45 ˚C. After 3% culture broth was transferred in 300 ml of PM and pH adjusted to; 4, 5, 6, 7 and 8. Samples were collecting at 12, 16, 20 and 24 hours. Also, the culture broth were diluted and spread-plated for cells count, while the supernatant or filtrate culture was assayed for mannanase activity.

Optimization culture condition for mannanase production

Experimental design

Taguchi techniques are mathematical modeling based on orthogonal array)OA(. Using OA to reduce the number of experiment and to identify significant factors. Taguchi method was applied to determine the parameters, which significantly influence the mannanase production by *Bacillus circulans* NT 6. 7 in a batch fermenter. The parameters of mannanase production were optimized using the standard orthogonal L9 arrays to determine culture condition as mannanase values to investigate three independent variables of inoculum concentration, agitation rate and aeration rate for three different levels)1, 2, 3(as shown in Table 1. The Taguchi with the creation of a series of experiments utilizing Minitab 16 Software **) Minitab Inc., USA(** . In this study, the condition for mannanase production was based on the L9 array when, L is Latin square array and 9 is the number of experiment run flowed in Table 2.

Table 1 Culture condition parameters and assigned levels selected for optimization

Parameter	Level		
	1	**2**	**3**
Inoculum (%); A	0.1	0.5	1.0
Agitation (rpm); B	200	400	600
Aeration (vvm); C	0.5	0.75	1.00

Table 2 Taguchi L9 orthogonal array of experimental design.

Experiment number	Control factor			Parameter value		
	A	**B**	**C**	**Inoculum (%)**	**Agitation (rpm)**	**Aeration (vvm)**
1	1	1	1	0.1	200	0.50
2	1	2	2	0.1	400	0.75
3	1	3	3	0.1	600	1.00
4	2	1	2	0.5	200	0.75
5	2	2	3	0.5	400	1.00
6	2	3	1	0.5	600	0.50
7	3	1	3	1.0	200	1.00
8	3	2	1	1.0	400	0.50
9	3	3	2	1.0	600	0.75

Mannanase production

Fermentation experiments were performed for mannanase production by *Bacillus circulans* NT 6.7 employing the selected 9 experimental trials)Table 2(. All experiments were conducted in 5-l fermenter) Biostat-B(containing 2 L of mineral salt medium and control temperature at 45 ºC.

Determination of mannanase activity

Mannanase was assayed by measuring the reducing sugars using dinitrosalicylic acid)DNS(method, adapted from **Miller (1959(**. The mannanase assay mixture contained 0.1 ml of 1%)w/v(locust bean gum prepared in 50 mM potassium phosphate buffer, pH 6 and 0.1 ml of diluted culture broth. The reaction mixture was maintained at 50 ºC for 60 min. After incubation, 0.2 ml of DNS reagent was added and boiled for 5 min, cooled and diluted to 2 ml. The absorbance was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme to liberate 1 µmol of mannose per minute under the assay conditions. Controls were routinely included in which enzyme preparation or substrate was omitted.

RESULTS AND DISCUSSION

Development of culture medium

A preliminary study of mannanase production by *Bacillus circulans* NT 6.7 used producing enzyme medium showed very higher cost than mineral salt medium. Mannanase was produced by *Bacillus circulans* NT 6.7 using copra meal and defatted copra meal for carbon source. It was suggested that, mannanase activity at 24 hours of producing medium supplement with copra meal and defatted copra meal were 0.24 U/ml and 4.79 U/ml, respectively **)Pangsri, 2014(**. In addition, test in mineral salt medium supplement with copra meal and defatted copra meal were 0.002 U/ml and 7.26 U/ml, respectively. The results indicate that, defatted copra meal was a good carbon source for mannanase enzyme production. This is because defatted copra meal contained high amount of carbohydrate but, low fat content. Fat might be the cause of cell growth inhibition observing in copra meal medium. This result related with previous findings by **Lin and Chen)2004(**, who suggested that the presence of coconut oil actually inhibited mannanase production from *Aspergillus niger* NCH-189. Consequently, the defatted copra meal was selected for mannanase enzyme production in fermenter cultivation.
Moreover, mineral salts medium supplemented with defatted copra meal (1%) showed culture growth and the enzyme activity higher than in the producing medium supplemented with defatted copra meal (1%) (9.44 and 8.88 Log CFU/ ml) and (13.88, 5.56) U/ml at; 45ºC, 18 hours, respectively. Upon which the activity of the enzyme curve determined as shown (Figure 1).

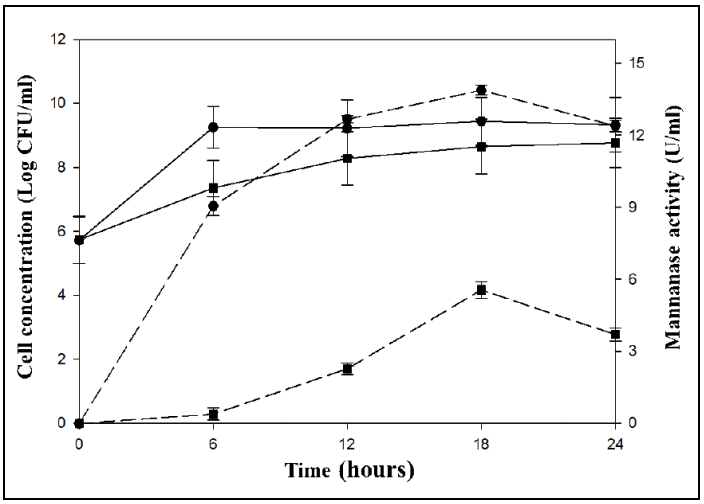

Figure 1 Relationship between microorganism growth and enzyme activity over time in producing medium)■—■;▲—▲(and mineral salts medium)●—●,▼—▼(.

The effect of defatted copra meal on culture and activity vary depending on the concentration (Table 3). Concentration of 1.5% showed the highest growth and enzyme activity (10.51±0.53 Log CFU/ml and 12.84±4.38 U/ml), followed by 2.0% (9.77±0.52 Log CFU/ml and 11.50±2.63 U/ml), 1.0% (9.80±0.71 Log CFU/ml and 10.33±3.17 U/ml) and 0.5% (9.65±0.78 Log CFU/ml and 7.20±1.43 U/ml) showing the lowest, respectively. The effect was more toward enzyme activity than the culture growth. Thus, 1.5% concentration of defatted copra meal was chosen to study in fermenter for enzyme production.

Microbial growth and enzyme production in fermenter using mineral salts medium supplement with defatted copra meal was compared with that of the shake flask to appreciate the different patterns. This is because the conditions for growth and enzyme production in fermenter could be controlled aeration rate and more control agitation rate. The results shows that, 2.0% defatted copra meal gave the highest activity (32 U/ml) (Figure 2) and therefore, selected for the enzymes production. The defatted copra meal at concentration 1.5% also shows

higher enzyme activity than 1.0% but, the enzyme activity was found slightly different from 2.0% concentration. The concentration of defatted copra meal affected the enzyme production because defatted copra meal was inducer of the enzyme activity.

Table 3 Microorganism growth, enzyme activity and yield in mineral salts medium using defatted copra meal at concentrations of 0.5%, 1.0%, 1.5% and 2.0% as carbon source compared with 1% defatted copra meal in producing medium (control).

Defatted copra meal (%)	Time (hr.(	Growth (Log CFU/ml)	Activity (U/ml)	$Y_{p/x}$ (U/mg)
0.5	0	4.64±1.10	0.05±0.00	
	6	8.45±0.72	6.23±3.21	22.67±13.23
	12	8.91±0.15	7.34±2.91	24.08±12.11
	18	9.65±0.78	7.20±1.43	20.48±10.30
	24	8.69±0.05	6.25±1.97	24.71±16.13
1.0	0	4.66±1.10	0.04±0.01	
	6	9.09±0.17	7.16±3.87	22.70±14.57
	12	9.44±0.41	8.87±4.42	27.63±20.32
	18	9.80±0.71	10.33±3.17	29.57±18.91
	24	9.31±0.02	10.26±3.00	33.72±19.30
1.5	0	4.66±1.10	0.05±0.00	
	6	8.98±0.22	6.10±4.12	19.09±12.70
	12	9.73±0.30	11.49±4.80	33.19±21.30
	18	10.51±0.53	12.84±4.38	31.26±18.14
	24	9.72±0.87	11.46±0.02	31.58±2.99
2.0	0	4.65±1.10	0.05±0.00	
	6	8.06±0.13	3.38±2.13	13.52±7.51
	12	9.07±0.09	11.39±4.67	36.75±20.40
	18	9.77±0.52	11.50±2.63	31.04±13.30
	24	9.12±0.00	12.09±2.06	41.09±19.70
1% (control)	0	4.97±1.37	0.06±0.01	
	6	7.74±0.18	0.35±0.05	1.56±0.83
	12	8.22±0.46	1.32±1.34	6.52±6.77
	18	8.50±0.55	4.16±1.97	16.44±11.06
	24	9.07±0.00	3.70±0.00	15.10±0.00

Note: $Y_{p/x}$ was expressed as yield equals to product value divided by cell dry weight.

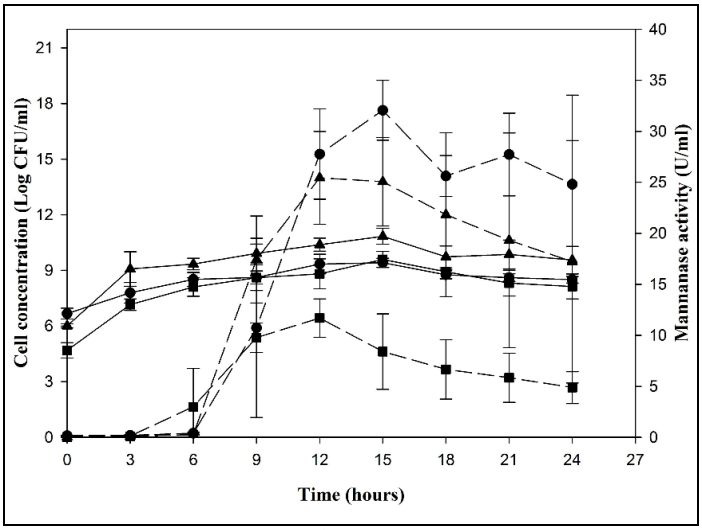

Figure 2 Microorganism growth in fermentor and enzyme activity with time in mineral salts medium using defatted copra meal as carbon source at varying concentrations)—■—,- -■- -) 1.0%,)—▼—,- -▼- -(1.5%, and)—●—,- -●- -) 2.0% inoculum, 600 rpm and 0.75 vvm **)Pangsri *et al.*, 2015(**.

Effect of temperature and pH on mannanase production

A preliminary study of mannanase production by *Bacillus circulans* NT 6.7 was cultivated on locust bean gum medium, pH 7.0 at different temperatures of 40, 45 and 50ºC, respectively. The result showed the highest enzyme activity at 45°C)2.35 U/ml(, the yield of production from cell was 0.62 U/CFU and the specific growth rate was 0.23 hr^{-1})calculated by viable cell count(. The result of *Bacillus circulans* NT 6.7 was similar to *Bacillus subtilis* 5H which produced mannanase at 45°C **) Khanongnuch *et al.*, 1999(** , but it was higher than mannanase production from *Bacillus sp*. KK01 at 30°C **) Hossain *et al.*, 1996(** and *Aspergillus niger* NCH- 189 at 30°C **)Lin and Chen, 2004(**.

The *Bacillus circulans* NT 6.7 was grown on locust bean gum medium at 45ºC in varying pH of 4.0, 5.0, 6.0, 7.0 and 8.0. The *Bacillus circulans* NT 6.7 could not grow at pH 4.0 and 5.0. At 20 hours, the highest mannanase activity was 2.41 unit/ml and the cell count was 7.93 Log CFU/ml at pH 6.0)data not shown(. The pH 6.0 was the best pH for mannanase production from *Bacillus circulans* NT 6.7. The result was similar to mannanase production from *Sclerotium rolfsii*, pH 6.0 **)Gübitz *et al.*, 1996(**, but lower than that reported for mannanase production from *Aspergillus niger*, pH 7.0 **)Ademark *et al.*, 1998(** and *Bacillus subtilis* KU-1, pH 7.0 **)Zakaria *et al.*, 1998(**. This study used locus bean gum because it's a common substrate used in mannanase industrial production. The structure of locus bean gum is galactomannan same with copra meal but the copra meal is complex structure it's difficult to hydrolyze more than locus bean gum. However, Next study change to used defatted copra meal to produce mannanase because want to decrease cost of medium.

Optimization culture condition for mannanase production

The experiment was to assess the fermentation conditions in fermenter using three) 3(factors; inoculum, stirring speed and aeration rate at 45ºC. This is because using different temperatures, preliminary study showed the highest enzyme activity and highest growth at 45ºC. Moreover, pH during fermentation was not control because it falls within pH 6-7. At this range, the bacteria growth and the release of product are not significantly affected. Using the experimental design Taguchi L9, it was found that, the main factor affecting fermentation was the concentration of *Bacillus circulans* NT 6.7, followed by the stirring speed and aeration rate. It was found that the best conditions to obtained higher enzyme production in the fermenter was the concentration of 1.0% inoculum, agitation rate of 600 rpm and aeration rate of 0.75 vvm at 15 hours. Thus, the maximum enzyme activity obtained at this condition was 29.57 U/ml)Table 4(, which is higher than the prediction value.

Table 4 Optimal conditions at varying cultivation time using Minitab 16 program with Taguchi L9 experimental design

Optimum condition					
Hr.	**Factor**	**Level**	**Expect value**	**Experimental value**	**Error (%)**
12	inoculum STIRR Aeration	1% 600 rpm 0.75 vvm	19.13 U/ml	24.41 U/ml	27.60
15	inoculum STIRR Aeration	1% 600 rpm 0.75 vvm	24.15 U/ml	29.57 U/ml	22.44
18	inoculum STIRR Aeration	1% 600 rpm 0.50 vvm	27.86 U/ml	24.66 U/ml	-11.49
21	inoculum STIRR Aeration	1% 600 rpm 0.50 vvm	33.90 U/ml	27.71 U/ml	-18.26
24	inoculum STIRR Aeration	1% 400 rpm 0.50 vvm	33.37 U/ml	27.41 U/ml	-17.86

The program predicted 3 conditions to give the highest activity within the period of 12-24 hours. The results showed the optimum point of the enzyme at different time intervals) Figure 3(, period where difference were obtained to allow comparison. The condition at the 15 h time only showed higher activity values than predictive values, which approve the maximum product value. This is because the relationship between time and mannanase activity shown in figure 3 indicate a trend similar to the observation attained in the experimental results according the program)Table 4(.

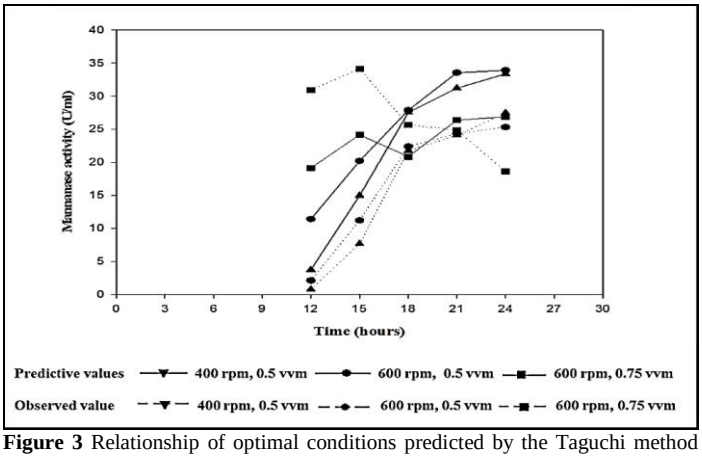

Figure 3 Relationship of optimal conditions predicted by the Taguchi method compared with actual values of enzyme activity in a fermenter at 12-24 hours.

The enzyme activity of the experiment and predicted values evaluated were dependable at the confidence interval of 95% based on the experiment data. This was used to build a linear regression model to describe the relationship between the study variables that affects the activity of enzymes. The correlation coefficient)R^2(was 0.75 as shown in the equation.

$$Y = -13.809 + 19.715X_1 + 0.021X_2 + 4.240X_3$$

Where X_1 is a concentration of inoculum)%(, X_2 is the rate of stirring)rpm(and X_3 is the rate of aeration)vvm(.

A regression model was used to calculate predicted values of mannanase activity compared with experimental values. Statistical analysis showed that regression was not significant)P<0.05(. The model may not be good theoretically, because the correlation coefficient indicates the goodness of fit for the model. This equation showed the lowest value of correlation coefficient may be have another model its better. Conversely, this study suggested the model as best, because the condition provided maximum yield at surface constraints. Inoculum was not increased more than 1% which was the concentration limitation due to the high rate of exponential phase. Agitation rate were not increased more than 600 rpm respectively because the limitation of fermenter in the large scale cannot working. However, the factor of time it important for enzyme production but can't know the maximum activity range. This study don't designated time in Taguchi.

The results of this experiment indicated that the bacteria increased in number at 15 h, thus resulted in high growth of *Bacillus circulans* NT 6.7 with desire mixing and aeration. Higher population led to more products liberation.

Table of regression Analysis at 15 hours

Predictor	**Coef**	**SE Coef**	**T**	**P(<0.05)**
Constant	-13.809	9.867	-1.40	0.221
Inoculum	19.715	5.637	3.50	0.017
Agitation rate	0.02062	0.01271	1.62	0.166
Aeration rate	4.24	10.17	0.42	0.694

Following Taguchi design, 2% defatted copra meal concentration in the fermenter resulted in 32.07±2.94 U/ml of mannanase activity under the optimum condition in 9 run experiment)table 2(. Upon repeating)3 times(under the same optimized condition in the fermenter, the mean of activity was 29.57±4.81 U/ml, which was not significantly different. The concentration)1.0%(of inoculum, 600 rpm agitation, and 0.75 vvm aeration rate at 45ºC for 15 hours gave the highest enzyme activity of 29. 57±4. 81U/ ml) Figure 4(, which corresponds to the observation by **Pangsri)2014(** and **Feng *et al.*)2003(** . It was found that, the maximum enzymes production by *Bacillus circulans* NT 6.7 was attained using producing medium containing 1% defatted copra meal as a carbon source.

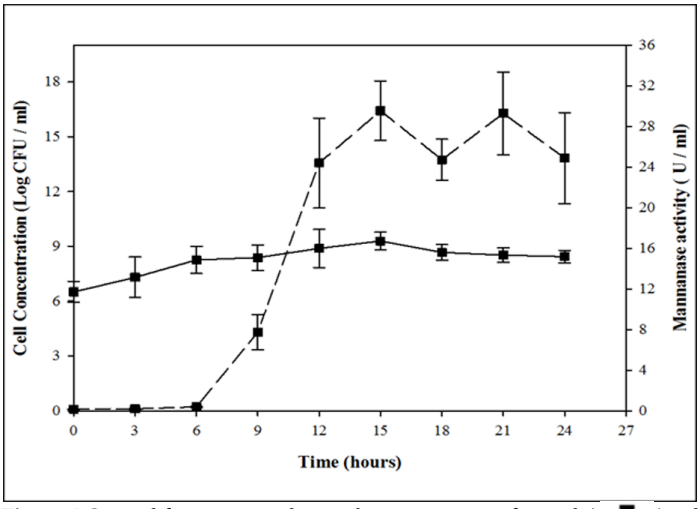

Figure 4 Optimal fermenter condition showing pattern of growth)—■—(and enzyme activity)▪ ■ ▪(.

In the fermenter containing mineral salts medium, maximum enzyme activity of 27.70 U/ml was obtained at 6 h. This was higher than the activity value observed using the Producing medium. Though, using the producing medium maximum enzymes activities can be achieved 9 hours lower than in the current study. This possibly because of the presence of poly peptone and corn steep liquor, which is easily used by the bacteria for their growth and activity. Thus, this could have resulted in maximum activity at shorter time than in current study. However, using producing medium for enzyme production come with challenges such as enzyme activity in each production is fixed depending on corn steep liquor used, and high price medium composition.

The study compared optimum condition in the enzyme production and beta-mannanase activity to previous work by **Feng *et al.*)2003(**, which used *Bacillus licheniformis* NK-27. From the former study, optimal conditions to achieved maximum enzyme yield were 600 rpm stirring rate and 0.75 vvm aeration rate at 30ºC for 36 hours. However, in current study, using mineral salts medium, maximum enzyme production can achieved similar stirring rate and aeration by *Bacillus circulans* NT 6.7 at less time)15 h(. Hence, current finding could be considered as best for enzyme production in the industrial scale.

CONCLUSION

Mineral salts medium supplemented with defatted copra meal as carbon source can be used to produce the enzyme beta-mannanase from *Bacillus circulans* NT 6.7. In the shaking flask, 1.5% concentration was suitable to obtain the maximum bacteria growth of 10.51±0.53 Log CFU/ml and enzymes activities of 12.84±4.38 U/ml at 18 hours. Meanwhile, using fermenter, 2% concentration showed the highest bacteria growth of 7.94±0.41 Log CFU/ml and the highest enzyme activities of 32.07±2.94 U/ml at 15 hours. Considering this, optimization of the enzymes production in a reaction tank 5 liters)fermenter(using working volume of 2 liters at 45ºC was determined. It was found that, the optimum condition to achieved higher enzyme activity) 29.57±4.81U/ml(were 1.0% inoculum concentration of bacteria, 600 rpm agitation and 0.75 vvm aeration rate at cultivation time at 15 hours. The study suggest that, mineral salts medium might be good to achieve higher enzyme production at low cost. Therefore, this could be considered to be used in the industrial scale.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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