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Optimization and Scaling-up of Cyclodextrin Glucosyltransferase Production by the Local Isolate *Bacillus paramycoides* MZ005634

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Abstract

Cyclodextrin glycosyltransferase is an important industrial enzyme that converts starch in to cyclodextrins (α-, β-, and γ-CDs). In this study our aim was to optimize the activity of produced CGTase for the production of β-CD from a local bacterial isolate and up-scaling the fermentation process. A terrestrial *Bacillus paramycoides* was isolated from soil obtained from sewage of Ismailia canal and identified by 16S rRNA analysis for the production of CGTase enzyme. Fractional factorial design (FFD) was applied to study the influence of the tested variables and analyze the relationship between them. The results represent an improvement in CGTase activity of 1.10 folds when compared to that previously obtained using the basal medium under the same cultivation conditions. The generated model was found to be very adequate for CGTase activity (81.57% accuracy) as the experimental value was compared to the predicted value. Upon applying a series of batch fermentations with different agitation rate an increase in CGTase activity about 1.6 folds compared to that obtained using shaked flasks.

Keywords: Beta-cyclodextrin; fractional factorial design (FFD); fermenter.

1. Introduction

Cyclodextrins are cyclic non-reducing oligosaccharides which are composed of six (cyclomaltohexaose, α-CD), seven (cyclomaltoheptaose, β-CD), and eight (cyclomalooctaose, γ-CD glucose molecules [1]. The difference between the three forms is the polar cavity size and their solubility in water. However, the β-form is the most important in practical use. The unique structure of the torous-shaped cyclodextrin shows the hydrophobic CH groups on the inside of the ring structure and the hydrophilic OH groups on the outside. This helps CDs to form inclusion complexes with various organic and inorganic compounds. This function is adopted in many applications such as medicines, food, agricultural, chemicals, cosmetics and pharmaceutical industries [2,3].

Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) is a key microbial industrial enzyme that catalyzes the cleavage of the glycosidic bond between two or more carbohydrates or between carbohydrates and non-carbohydrate moiety [4]. Four different reactions namely coupling, cyclization, weak hydrolysis and disproportionation are catalyzed by CGTase in the process of cyclodextrinsformation[5].

CGTase is an extracellular enzyme produced by bacterial genus namely Bacillus; B circulars [5]. B. marcerans [4], B. cereus [6]. B. stearothermophilus HRi [7]. Other CGTase producing species were Klebsilla pneumoniae AS-22[8], Brevibacterium spp. [9] and Mycobacterium terrae KNRq [10]. For the industrial enzyme production, the fermentation

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process is employed by different microorganisms under controlled conditions [11].

In the fermentation process, the physical and biological parameters influence the enzyme yields [12, 13]. Studying one factor at a time for the optimization of the enzyme is a time-consuming and difficult technique because it neglects the combinatorial effects [14,15]. Fractional factorial design (FFD) is especially suitable to account for the interactions and identify the most significant components in the medium formula [16].

Beside enzyme activity optimization, scaling up its production is important for developing the same fermentation efficacy, maintaining similar product yield and certify reliable product quality as obtained in the small-scale fermenter at attractive economical values. Thus, Batch and Fed-batch CGTase production was reported using free and immobilized cells [17, 18, 19, 20, 21, 22, 1].

The main objectives of this work were to isolate a CGTase producing isolate (Bacillus sp.) from Egyptian soil and molecularly identify it and optimize enzyme production using fractional factorial design (FFD) experiment to develop a mathematical model between the significant factors for the optimum production of CGTase from the chosen isolate. Partial purification of the enzyme was also done using ammonium sulphate precipitation. Furthermore, production of the extracellular CGTase using batch technique was performed.

2. Experimental

2.1. Materials

Soluble starch, yeast extract, and peptone were obtained from Qualigens, India. Phenolphthalein was purchased from Merck India Ltd. All other chemicals used were of analytical grade (Difco).

2.2. Bacterial strain

Bacterial strainswere isolated from sewage of Ismailia canal, Ismailia, Egypt. One gram of soil samples was serially diluted to 10^{-4} in distilled water, shacked and cultured in Nutrient agar medium containing fluconazole 150 mg/L as antifungal. The plates were incubated at 30^0C for 24 hours then stored at 4^0C with monthly subculture maintenance. The isolated strains were identified by biochemical activity (data not shown) and by 16S ribosomal RNA (rRNA) gene sequencing analysis. Pure bacterial isolates were preserved in cryotubes containing 2 ml of 20% glycerol and stored at -80^0C and subcultured on nutrient agar slants and kept at 4^0C.

2.3. Molecular identification

A pair of universal primers 16S27FWD (5'AGAGTTTGATCMTGGCTCAG 3') and16S1492RVS (5'TACGGYTACCTTGTTACGACTT3') were used to amplify 16S RNA. The sequencing was carried out using Maxima Hot Start PCR Master Mix (Thermo K1051) by Sigma Co. (Egypt). The 16S rRNA sequence was used as query in Blast search against National Center of Biotechnology Information (NCBI) database. MEGA6 software [23, 24]was used to construct an evolutionary model and to generate the maximum phylogenetic tree. The 16S rRNA gene sequence was submitted to the NCBI Genbank with nucleotide sequence database under an accession number.

2.4. Inoculum preparation

Bacterial isolates were sub-cultured on nutrient agar slants and kept at 4^0C, from these slants inoculums were done where whole slant was transferred into Erlenmeyer flasks containing 20 ml nutrient broth medium and incubated 24hrs at 37^0C before transferring in to production medium.

2.5. CGTase production medium

After bacterial isolation and purification, 3mL (v/v) of bacterial inoculums was inoculated into Erlenmeyer flasks containing 100 mL of the production medium of the following composition (g/L): soluble starch, 12.5; peptone, 1.5; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.2 and $FeCl_3$, 0.02 at pH 6.5. The bacterial culture was incubated at 30^0C in an incubator shaker for 1, 2 and 3 days. At the end of the incubation period, 1ml of the culture medium was removed and separated by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was assayed for CGTase activity and used as enzyme solution.

2.6. CGTase assay

CGTase activity was determined according to the modified method reported by Savergave *et al.*[18]as follows: the reaction mixture containing 40 mg of soluble starch (Sigma) in 1.0 mL 50 mmol L^{-1} phosphate buffer (pH 6.5) and 1.0 mL of the enzyme solution were incubated at 60 °C for 20 min. The reaction was stopped by the addition of 3.5 mL of 30 mmol L^{-1}NaOH solution, and then 0.5 mL 0.02% (w⁄ v) phenolphthalein solution prepared in 5 mmol L^{-1} Na_2CO_3 was added. After standing for 15 min at room temperature, the absorbance at 550 nm was read. The standard β-CD estimation was also carried using the same method. One unit (U) of enzyme activity was defined as the amount of enzyme that produced one µmole of β-CD per minute under the assay conditions.

2.7. Total protein content

The total protein content was determined according to Lowry *et al.* [25] method with bovine serum

albumin as the standard. The protein content was calculated by checking the absorbance at 750 nm.

2.8. Statistical analysis for experimental design

In order to optimize the CGTase activity, fractional factorial design (FFD) for six independent variables was adopted. The experimental design was based on Minitab software version 16.1.1 (**Minitab Inc. USA. 2020**). FFD was used to obtain the combination of values that can maximize the response within the region of the three-dimensional observation spaces and thus helps to design a minimal number of trials [26,27]. The production of CGTase was evaluated by T statistics and analysis of variance (ANOVA). A 3 level factorial design was employed to evaluate the individual and combined effects of the six variables (soluble starch, fermentation time, peptone, KH_2PO_4, inoculum size and pH). A significant level of 5% was used as the criterion to reject the null hypothesis.

According to the applied design, 10 combinations were executed **(Table 1),** and their observations were fitted to the following first order polynomial model:

$$Y= b_o + b_1X_1 + b_2X_2 - b_3X_3 + b_4X_4 + b_5X_5 + b_6X_6$$

Where Y is the dependent variable; X_1, X_2, X_3, X_4, X_5 and X_6 are the independent variables as mentioned above; $b_o$is the regression coefficient at the center point; b_1, b_2, b_3, b_4, b_5 and b_6 are linear coefficients. The quality of the fit of the polynomial model equation was expressed as the coefficient of determination, R^2. All trials were performed in triplicate, and the average β-CD yield observations were considered the responses. Regression analysis was applied on the results to evaluate the effective factors.

The coefficient of determination (R^2), adjusted coefficient of determination (R^2-adj), and predicted coefficient of determination (R^2-pred) were used to compare the models. R^2 values close to 1, means the model is more accurate [28]. After selecting the most accurate model, the analysis of variance (ANOVA) was used to study the statistical significance of the regression coefficients by conducting Fisher's (F-test) at 95% confidence level.

Validation model

It was performed under conditions predicted by the experiment model. The experiments were examined in triplicate at an interval of 2 days.

2.10. Fermenter set-up

All cultivation processes in the present set of experiment involved estimation of β, cyclodextrin at different stirring rate (200, 300, 400 rpm) and were carried out using benchtop lab scale 5L fermenter Braun Biotech International, Germany) at working volume 3l. The initial cultivation medium previously mentioned was sterilized at 121^0C for 15 min. and sterilization of the fermenter was carried out by autoclaving for 20 min. Batch fermentation media was inoculated with 5% (v/v) bacterial inocula. Cultivation was carried out at 30^0C at different time intervals (6,12,24,36,48,60,72, and 84hrs) for quantitative estimation of β cyclodextrin [29**].**

2.11. Biomass determination

After fermentation, the biomass was separated from the supernatant by centrifugation at 5000 rpm for 30 min. at 4^0C. The biomass was re-suspended in 5 mL distilled water and centrifuged again for cell washing. After removing the supernatant, 30 ml of distilled water was added and the precipitate was re-suspended. Then the optical density was measured at 600 nm and biomass was quantified by comparing with the standardized curve based on dry mass versus optical density.

3. Results and Discussion

3.1. Screening of CGTase producing microorganisms

The CGTase producing microorganisms were isolated on nutrient agar medium supplemented with fluconazole 150mg/L as an antifungal. As shown in Fig. (1) the cell free supernatant of the eight isolates (B1-B8) were tested for CGTase production after 24, 48 and 72 hrs. the highest CGTase activity of the crude enzyme was expressed by the isolate no. B1 (270± 1.8 U/mL) after 24 hrs.

According to previous reports, different species of *Bacillus* have shown same cultivation period [22, 3,4]. On the other hand, Ahmed and El-Refai [30] reported that 72 hrs was the best incubation period for maximum enzyme production.

However, Arce-Vazquez et al. [31] reported highest enzyme activity after 36 hrs using *B. megaterium*. Ramli et al. [32] stated that highest enzyme production was expressed at 32 hrs of fermentation using *Bacillus sp.* And decreased thereafter.

The reason for this might be due to the depletion of the nutrients or denaturation of the enzyme caused by the interaction with the medium components or might also be due to change in medium pH [33].

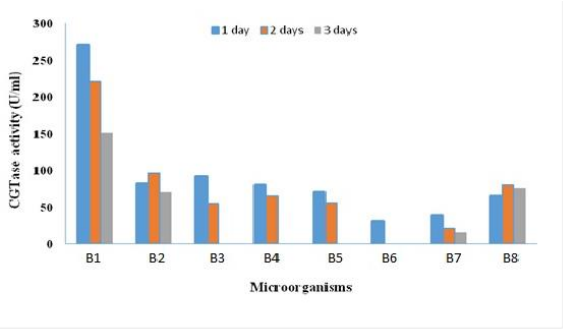


Fig. 1. CGTase activity exhibited by different bacterial isolates at different fermentation tim

3.2. Molecular identification of Cyclodextrin glucosyl transferase Producing strain

Since Isolate (B1) showed the highest activity therefor it was selected for molecular identification using 16S rRNA. Blast search at the NCBI database indicated that the 16S rRNA sequence of bacterial strain B1 is 98-99% identical to 16S rRNA sequences of Bacillus species indicating that the strain B1 belongs to this bacterial genus. Phylogenetic analysis showed 99.8% similarity between *Bacillus sp.* (B1) and *Bacillus paramycoides* (accession number MT332160.1) (Fig. 2). The sequence data was submitted in the Genbank and *Bacillus sp*. (B1) was given accession number MZ005634.This is in accordance with previous reports that *Bacillus* species are the well-known natural CGTase producers [34]. The bacterial CGTase was known as a multifunctional enzyme produced by different bacteria such as *B.megaterium, Klebsillaoxytoca, Micrococcus spp* [35].

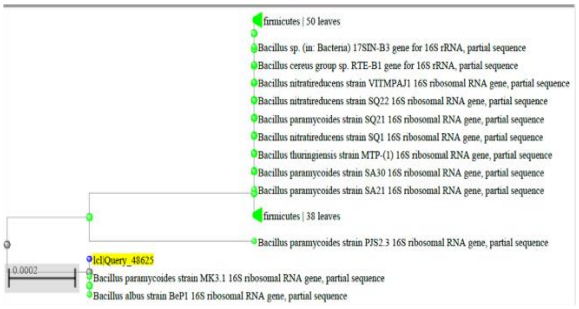


Fig. 2. Neighbor-joining phylogenetic tree based on 16 rRNA gene sequences showing the relationship between bacterial isolate B1 and the species *Bacillus*

3.3. Statistical optimization of cultural conditions for CGTase activity by fractional factorial design (FFD)

The experimental matrix with six variables (soluble starch, peptone, KH_2PO_4, pH, time and inoculum size) was designed and the corresponding results were presented in Table (1) The activity of CGTase produced by *Bacillus paramycoides* MZ005634 varied from 135.24 to 298.24 U/mL in the 10 trials which reflected the importance of the medium composition as well as the environmental factors to attain high enzyme production [36]. The main effect of each factor is shown in Table (2) where incubation time followed by peptone have positive concentration effect while other variables had insignificant effect on the enzyme production. It should be noted that either positive or negative concentration effect of a variable is important as this guides whether to increase or decrease the concentration of a variable in the optimization of the medium [10].

All of the terms regardless of their significance were included in the first order polynomial equation as follows:

β-CD activity $= 226.6 + 37$ Time $+ 18$ starch $- 19.4$ Inoculum size $+ 21.1$ peptone $+ 10.4$ pH $+ 9.9$ KH_2PO_4

The determination coefficient (R^2) of the model was found to be 81.57% which is a value of > 75% indicating the fitness of the model. This means that the study indicates 81.57% of the variation in the enzyme activity was attributed to the independent variables while 18.43% of the total variance could not be explained by the model. The adjusted R^2 was 44.7% which accounted for the predictor's number in the model. Both the predicted R^2 and the adjusted value suggested that the model fitted the data. ANOVA analysis (Table 3) showed that the model F-value was 2.21 and corresponded to P-value 0.275 which indicated that the model was both well fitted and significant. The larger the magnitude of the t-value and the smaller the P-value, the more significant the corresponding factor [4]. From the effects of the first order in Table (2), it concluded that the time had the most significant effect on CGTase activity. In the same manner, the quadratic main effect of the size of the inoculum ($P < 0.081$) was also significant. Similarly, incubation period was among the parameters that showed positive effect on activity of CGTase produced as stated by Wadetwar et al. [37]. Rosso et al. [38] also found that CGTase production from *B. circulans* time course showed a characteristic late-log production/secretion profile which is not related to growth where enzyme production increased between 24 and 48 hrs culture. The statistical optimization resulted in about 1.1-fold increase in CGTase activity

produced by *B.paramycoides* MZ005634 compared to the non-optimized medium. These observations clearly suggested that the nutritional and physical requirements of the microbes differ from one another and therefore, need to be optimized for each strain.

Table (1) Factorial regression: CGTase enzyme activity versus different fermentation factors

Run	Time (X1)	Starch (X2)	Inoculum (X3)	Peptone (X4)	pH (X5)	KH_2PO_4 (X6)	Activity(U/mL)
1	24	20	15	0.1	6	0.2	163.36
2	48	10	5	0.1	6	0.2	225.66
3	24	20	5	0.1	8	0.1	188.60
4	24	10	5	0.3	8	0.2	228.98
5	36	15	10	0.2	7	0.15	270
6	36	15	10	0.2	7	0.15	268
7	24	10	15	0.3	6	0.1	135.24
8	48	10	15	0.1	8	0.1	202.20
9	**48**	**20**	**5**	**0.3**	**6**	**0.1**	**298.24**
10	48	20	15	0.3	8	0.2	285.8

Table (2) Main effect of tested factors on produced CGTase enzyme activity

Term	Effect	Coeff	SE Coeff	95% CI	T-Value	P-Value	VIF
Constant		226.6	12.8	(185.9,267.3)	17.72	0.000	
Time	73.9	37.0	14.3	(-8.5, 82.5)	2.58	0.081	1.00
Starch	36.0	18.0	14.3	(-27.5,63.5)	1.26	0.297	1.00
Inoculum	-38.7	-19.4	14.3	(-64.9, 26.1)	-1.35	0.269	1.00
peptone	42.1	21.1	14.3	(-24.5, 66.6)	1.47	0.237	1.00
pH	20.8	10.4	14.3	(-35.1, 55.9)	0.73	0.520	1.00
KH_2PO_4	19.9	9.9	14.3	(-35.6, 55.4)	0.70	0.537	1.00

SE: Standard error. R^2 =R Squared = 81.57% (Adjusted R Squared = 44.70%)

Table (3) ANOVA of the fractional factorial model for optimization of different fermentation factors

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value
Model	6	21718.6	81.57%	21718.6	3619.8	2.21	0.275
Linear	6	21718.6	81.57%	21718.6	3619.8	2.21	0.275
Time	1	10931.3	41.05%	10931.3	10931.3	6.68	0.081
starch	1	2589.1	9.72%	2589.1	2589.1	1.58	0.297
Inoculum	1	2998.5	11.26%	2998.5	2998.5	1.83	0.269
peptone	1	3546.5	13.32%	3546.5	3546.5	2.17	0.237
pH	1	862.8	3.24%	862.8	862.8	0.53	0.520
KH_2PO_4	1	790.4	2.97%	790.4	790.4	0.48	0.537
Error	3	4907.7	18.43%	4907.7	1635.9		
Curvature	1	4492.7	16.87%	4492.7	4492.7	21.65	0.043
Lack-of-Fit	1	413.0	1.55%	413.0	413.0	206.50	0.044
Total	9	26626.3	100.00%	Total			

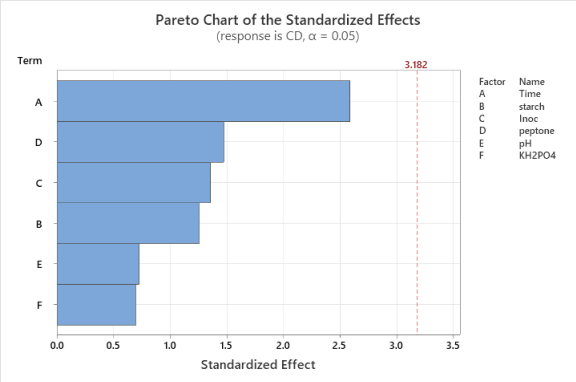


Fig. 3. Pareto chart of six factors and their interaction standard effects on CGTase activity production

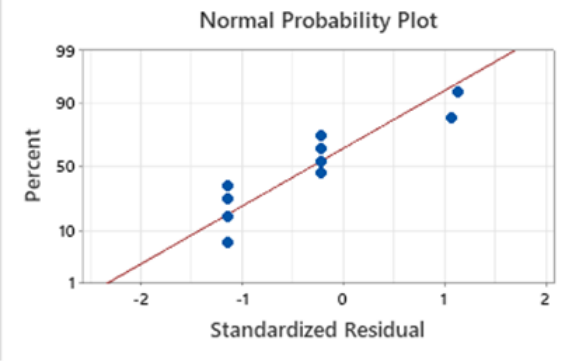


Fig. 4. Predicted Vs Residual plot

Validation of the model:

The accuracy of the model was studied (Fig. 4). The statistical analysis software was used to predict concentration of soluble starch (20g/L), incubation time (48 hrs), inoculum size (5% v/v) and CGTase activity (298.24 U/mL). The straight line of regression with data points across indicated the agreement between predicted and actual values as well as the suitability of the model. At the end of the optimization about 1.1-folds increase in the enzyme activity was achieved. These results indicated the validity of the applied enzyme.

3.4. Up-scaled fermentation for producingCGTase at different stirring rates

A set of experiments involved evaluation of β-cyclodextrin at different stirring rates (200, 300, 400 rpm) at the same fermentation conditions using bench-top lab scale 5L fermenter (Braun Biotech International, Germany) at working volume 3l. After culturing for 24hrs, a 300mL culture was inoculated into 3L working volume. The quantitative estimation of β-cyclodextrin was carried out at different time intervals (6, 12, 24, 36,48,60,72, and 84hrs) for each agitation rate.

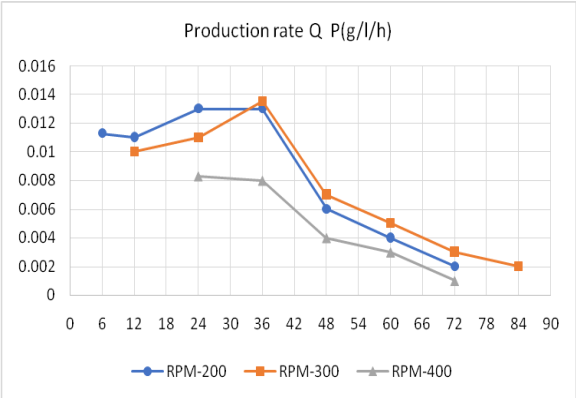
It was noticed that on stirring rate 200rpm for 24-36hrs, cell dry weight X_{max} increased gradually to reach its maximum level (6 g/L) as well as the enzyme activity (456 U/mL), volumetric production P_{max} (0.456g/L), specific production $Y_{(p/x)}$ (0.076g/g) and production rate Q_P (0.013g/L/h).

However, at stirring rate 300 for 36 h, all parameters were higher than those obtained at 200 rpm for the same time except for the cellular dry weight (4 g/L). Enzyme activity reached 485U/mL, volumetric production 0.485 g/g, specific production 0.08 g/g production rate 0.0135g/L/h.

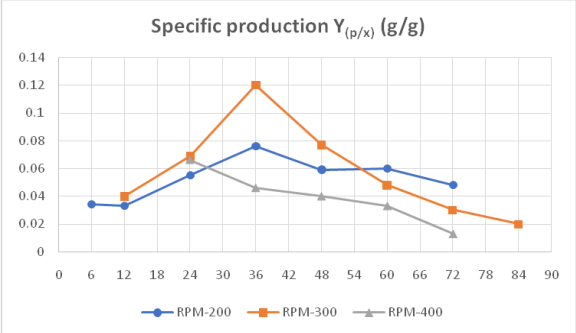
On the other hand, when fermentation was performed at higher stirring rate (400 rpm) for 36 h, all responses were decreased where maximum enzyme activity reached was 290 U/mL, cell dry weight 6.3g/l, volumetric production 0.29g/l, specific production 0.046 (g/g) and production rate 0.008 (g/L/h). This may be attributed to the high dilution rate accompanied by low enzyme activity [39].

These results agreed with that stated by Wang *et al.* [40] who found that on increasing the agitation speed, the activity of CGTase increased until it reached maximum at 200 rpm after which it started to decrease due to great shearing force that inhibited the growth of microorganism.

After scaling up in 5L fermenter with working volume 3.0L in batch cultivation, the enzyme activity increased up to 485 U/mL. The reason for the increase in enzyme activity may be due to the difference in hydrodynamic conditions, oxygen limitation in shake flask in addition to the shear rate [4]. Similar results were found by Yang e*t al.* [41] who found that CGTase activity reached 45.2 U/L in the scale up studies.



(a)



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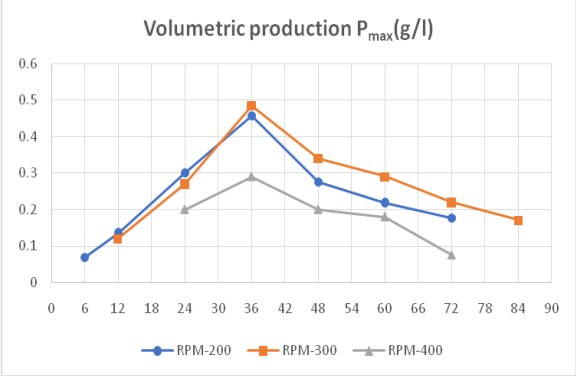

(c)

Fig. 5. Graph showing production time (hours) versus (a) CGTase enzyme production rate (g/l/h) (b) CGTase enzyme specific production (g/g) (c) CGTase enzyme volumetric production (g/l) at different agitation rates (rpm)

4. Conclusion

In the present study, initial screening for the highest CGTase enzymatic activity of 8 local bacterial isolates showed that isolate B1(*Bacillus paramycoides* MZ005634) after 24hrs of incubation expressed the highest activity (270 U/mL). Fractional factorial design (FFD) for statistical screening of the environmental and nutritional conditions required for optimization of the bacterial enzyme activity was done. Statistical optimization studies led to increase in enzymatic activity by 1.1-fold (298.24 U/mL). Up-scaling fermentation in a 5L fermenter was carried out using different agitation speed the best of which was 300 rpm where the cell dry weight was X_{mass} 6 g/L and the enzyme activity reached 485 U/mL. This study revealed the feasibility of using the local isolate and the enhancement of the CGTase activity (1.63 folds) when scaled-up in bench top fermenter.

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6. Conflict of interest

The authors do not have any conflict of interest.

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