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Research Article

PURIFICATION AND CHARACTERIZATION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM NOVEL MUTATED *BACILLUS* SP. TPR71HNA6

Kashipeta Ravinder*

Department of Biotechnology, College of Dryland Agriculture and Natural Resources, Mekelle University, Mekelle, Tigray, Ethiopia Phone: +251 919040957.

Corresponding author*: ravikamal999@gmail.com

ABSTRACT

Purification of cyclodextrin glycosyltransferase (CGTase) produced by a newly isolated mutated *Bacillus* sp. TPR71HNA6 was done in two steps using ammonium sulphate precipitation and chromatography and the purified enzyme was characterized by SDS-PAGE. Results indicated that the molecular weight was 75KDa. The purified enzyme was further characterized for optimum substrate concentration, pH, temperature, stability, metal ion effects, inhibitors and enhancers. After purification the specific activity of the CGTase increased from 97.51U/mg to 307.69U/mg and finally 30% was recovered with 3.15% purification fold.

Keywords: CGTase, *Bacillus sp.* TPR71HNA6, Purification, SDS-PAGE

INTRODUCTION

Various unit operations used in downstream processing for getting pure protein from fermentation broth constitutes the largest part of the production cost (Leaver *et al*., 1987). There are many reports on the purification of CGTase where it requires multiple steps of column chromatography after cell separation (Gawande *et al*., 2001; Martins and Hatti-Kaul, 2002). Purification strategies used mainly involve adsorption of CGTase on starch, followed by gel filtration. However, drawback with the starch column is that CGTase reacts with the starch and produces cyclodextrins during elution and thus required additional step to exclude the CDs. Due to the additional step of removal of CDs very low recovery of the purified CGTase is reported (Laxman *et al*., 2008). There are some techniques to eliminate these drawbacks. Among these was affinity chromatography on β-CD Sepharose gels, which has often been used for the purification of CGTase (Akimaru *et al*., 1991). In the present work, attempt is

made to purify CGTase produced by mutated TPR71HNA6 in two steps using Sepharose 4B gel affinity chromatography.

MATERIALS AND METHODS

All enzyme purification steps were carried out at 0 to 4°C.

Microorganism and Culture Conditions

A mutated *Bacillus* sp. TPR71HNA6 (Gen Bank No: FN993946) was used. This culture was stored in a nutrient agar slants and sub-cultured periodically once a week. The liquid samples were withdrawn and centrifuged at 10,000rpm for 10min to remove the biomass and other insoluble substrates from the culture. After centrifugation the supernatant liquid was collected and estimated for CGTase activity.

Estimation of CGTase activity

Enzyme activity was measured by decrease of phenolphthalein colour intensity. Enzyme assay was carried out according to the (Kaneko *et al.*, 1987) method. The reaction mixture containing 1ml of 40mg of soluble starch in 0.1M potassium phosphate buffer (pH 6.0) and 0.1ml of the crude enzyme from the culture was incubated in water bath at 60°C for 10min. The reaction was stopped with 3.5ml of 30mM NaOH. Finally, 0.5ml of 0.02% (w/v) phenolphthalein in 5mM Na_2CO_3 was added and mixed well. After leaving the mixture to stand for 15min at room temperature, the reduction in colour intensity was measured at 550nm. A blank lacking the enzyme is tested simultaneously with each batch of samples. One unit of enzyme activity was defined as the amount of enzyme that forms 1µgm of β-CD from soluble starch in 1min.

Ammonium Sulphate Precipitation

The crude broth obtained after fermentation was centrifuged at 6000rpm for 10min to remove the cell biomass. Solid ammonium sulphate was added slowly to the culture supernatant to get 60% saturation, stirred for 60min and left for overnight at 4^{o}C. The precipitate was harvested by centrifugation at 10,000 X g for 10 min, dissolved in 0.1M acetate buffer (pH 5.5) and dialyzed against same buffer overnight (4^{o}C). The dialyzed sample was then assayed for CGTase activity and protein content.

Separation of CGTase by affinity chromatography

Preparation of affinity matrix

The matrix was prepared according to the Sian *et al.*, (2005). Five grams of Sepharose 4B was thoroughly washed with distilled water. The gel was then transferred to 20ml coupling solution (0.1M NaOH) containing of 350mg β-cyclodextrin and reacted for 16h at 45^{o}C. After incubation, the gel was washed again with distilled water for 1h. Subsequently, the washed gel was transferred to methanolamine solution (pH 8.0) and was incubated for 24h at 50^{o}C. This step is essential to block any unreacted epoxy groups. The gel was then washed with 0.1M acetate buffer pH 4.0 followed by 0.1M Tris–HCl buffer pH 8.0 containing 0.5M NaCl. This cycle was repeated for three times. Finally, the gel was packed into a 15mm X 100mm column and equilibrated with two bed volumes of 0.1M sodium acetate buffer pH 5.5.

Affinity chromatography

The dialyzed enzyme mixture was loaded onto a 15mm X 100mm β-CD-Sepharose 4B affinity column that was previously equilibrated with the same

buffer at a flow rate of 25ml/h. The column was washed with 0.01M acetate buffer pH 5.5 for 4h. Elution of the bound enzyme was carried out with the same buffer supplemented with 1% β-CD. All fractions were collected and each one was assayed for CGTase activity and protein content. All CGTase purification procedures were performed at 4^{o}C.

Determination of molecular weight

The molecular weight of the purified enzyme was determined by SDS-PAGE, according to (Laemmli, 1970). A 15% cross linked polyacrylamide gel on a Tarson gel electrophoresis unit (Tarson, India) was used at constant voltage of 300V and 60mA for 90min at room temperature. Coomassie Brilliant Blue (0.1%) staining was used to detect the protein bands on the gel.

Characterization of CGTase

Effect of substrate concentration on CGTase activity

To observe the effect of substrate on CGTase the enzyme concentration was kept constant (0.1ml) and the various concentrations of starch (1 to 6g/L) were used. The CGTase activity was observed at different time intervals for each concentration of starch.

Determination of optimum pH and temperature

The optimum pH of the pure enzyme was determined by replacing 0.1M phosphate buffer pH 6.0 in the CGTase assay with the following buffers: citrate buffer, 0.1M (pH 4 to 6), phosphate buffer, 0.1M (pH 5 to 8), borate buffer, 0.1M (pH 8 to 10) and bicarbonate buffer (pH 9 to 11). The reaction was carried out using the CGTase assay procedure mentioned above. The optimum temperature of the pure enzyme was determined by performing the CGTase assay in different temperatures, ranging from 20° to 90°C at pH 6.0.

Determination of optimum pH and temperature by means of RSM

In order to determine the optimum pH and temperature of CGTase a central composite design was employed. These factors were coded at five levels starting from -1.414, –1, 0, 1, and 1.414 by using Eq. 1.

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad \text{---------- (1)}$$

where,

xi is the dimensionless coded value of the variable X_i, X_0 the value of the X_i at the centre point, and ΔX_i the step change.

For statistical calculations, the variables *Xi* were coded as *xi* according to Equation 1. The range and levels of the variables in coded units for RSM studies were reported in Table 3.8.The behavior of the system was explained by the following quadratic model 2.

$$Y = \beta_0 + \sum \beta_i * x_i + \sum \beta_{ii} * x_i^{\,2} + \sum \beta_{ij} * x_{ij} \quad \text{-------- (2)}$$

where,

Y is the predicted response, β_0 the intercept term, β_i the linear effect, β_{ii} the squared effect, and βij the interaction effect.

In the present investigation, 10 experimental central composite experimental designs was used to determine the optimum response of pH and temperature of CGTase produced by the *Bacillus* sp. MATLAB 7.0 (Mathworks USA) software was used for regression and graphical analysis of the data obtained.

Determination of pH and thermal stability

The pH stability of the enzyme was measured by incubating 0.1ml pure enzyme with 0.2ml phosphate buffer, 0.1M (pH 5 to 8) and borate buffer, 0.1M (pH 8 to 9) respectively at 60°C, without substrate for 30min. The remaining activity of the enzyme was assayed by the standard assay method. The temperature stability of the enzyme was measured by incubating 0.1ml pure enzyme with 0.2ml buffer (0.1M phosphate buffer, pH 6.0) without substrate at different temperatures (60℃–80°C) for 30min. Then the enzyme was cooled to room temperature and standard CGTase assay was performed to determine their residual activity.

The effects of metal ions and reagents on CGTase activity

Purified enzyme (0.1ml) was mixed with 0.2ml of 0.1 M phosphate buffer pH 6.0, containing different metals and reagents at 1 mM & 2mM (final concentration) and incubated for 10min at room temperature. The standard CGTase assay was performed to determine the activity.

Estimation of Kinetic parameters

The K_m and V_{max} values for the pure enzyme were determined by incubating 0.1ml of purified CGTase in 1ml 0.1 M phosphate buffer pH 6.0 at various concentrations of soluble starch solution, ranging from 1 to 6g/l at 60°C for 10min. The values of K_m and V_{max} were then determined by using the Graph pad Prism V 4.0 software. The activation energy was calculated using Arrhenius plot which was plotted ln V vs 1/T (K).

RESULTS AND DISCUSSION

Purification of CGTase

The crude CGTase obtained from the mutated *Bacillus* sp TPR71HNA6 was successfully purified to homogeneity in two steps (Table 1). The purified enzyme gave a single protein band on a SDS-PAGE gel (Fig. 1). The enzyme eluted from the β-cyclodextrin-bound Sepharose 4B column resulted in a 3-fold purification with a yield of 30%. As a result, the enzyme appeared as a homogenous band on SDS-PAGE (Fig. 1). However, as shown in Table 1, the yield of the purified CGTase from *Bacillus* sp was quite high compared to the literature. Tachibana *et al*., (1999) and Sian *et al*., (2005) employed α-CD-(epoxy)-Sepharose 6B affinity column to purify CGTase, thus they successfully purified the enzyme with a yield of 10% and 4% respectively.

Table 1: Purification of the CGTase from mutated *Bacillus* sp TPR71HNA6.

Fraction	Total activity	Total protein (mg)	Specific activity (U.mg-1)	Recovery (%)	Purification fold (%)
Crude	149000	1528	97.51	100	1
$(NH_4)_2SO_4$	40000	157	254.77	60.24	2.61
Sepharose 4B	12000	39	307.69	30	3.15

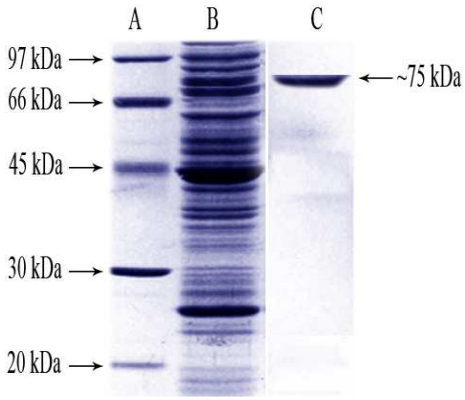

Figure 1: SDS-PAGE of purified CGTase from mutated *Bacillus* sp TPR71HNA6 Lanes: A) Molecular markers, B) Crude CGTase C) Chromatographic purified enzyme.

Molecular weight determination

Fig. 1 depicts the SDS-PAGE gel of the purified enzyme along with the crude enzyme. It was observed that the molecular weight of the purified CGTase was estimated to be 75kDa. The purified enzyme showed a single protein band on the 15% Coomassie Brilliant Blue stained gel which indicated homogeneity of the purified enzyme. Therefore, it can be concluded that CGTase is a monomeric protein. These results are in accordance with the previous results, CGTases from various *Bacillus* sp. had a molecular weight range of 68–88kDa. Nomoto *et al*., 1986 reported that CGTase from *Bacillus* sp HA3-3-2 has molecular weight 68kDa. The CGTase obtained from *B. autolyticus* 11149 has 70kDa molecular weight (Tomita *et al*., 1992). Fujita *et al*., (1990) observed that *Bacillus* sp. AL-6 secreted CGTase has 74 kDa MW. It was observed that β-cyclodextrinase from *B. firmus* has 78kDa (Gawande *et al*., 1999) and 80kDa (Sohn *et al*., 1997). However, Wang *et al*., (1995) reported CGTase from *Bacillus* sp. had a molecular weight of 38kDa, while Pongsawasdi and Yagisawa, (1988) and Martins and Hatti-Kaul, (2002) obtained large protein of 103 and 110kDa molecular weight from *B. circulans* and *Bacillus agaradhaerens*, respectively.

Effect of substrate concentration on CGTase activity

In order to study the influence of substrate concentration on the purified CGTase obtained from the mutated *Bacillus* sp TPR71HNA6, the enzyme was incubated at different time intervals with different concentrations of starch. The enzyme activity was analyzed by supplementation of varied starch

concentration ranging from 1 to 6g/L in the reaction medium. Fig. 2 shows the time and substrate profiles of CGTase activity. It is observed that highest activity of the enzyme was found at 4g/L starch concentration incubated for 30min. Further enhancement of the starch concentration did not alter the enzyme activity. In all the concentrations at 10 min interval the saturation point is attained after that a small increase in the activity was observed. Based on these observations it was inferred that the isolated CGTase follow the pseudo first order kinetics. At the higher substrate concentration levels (5 and 6g/L) the required incubation time to attain maximum activity was low when compared to the lower substrate concentrations.

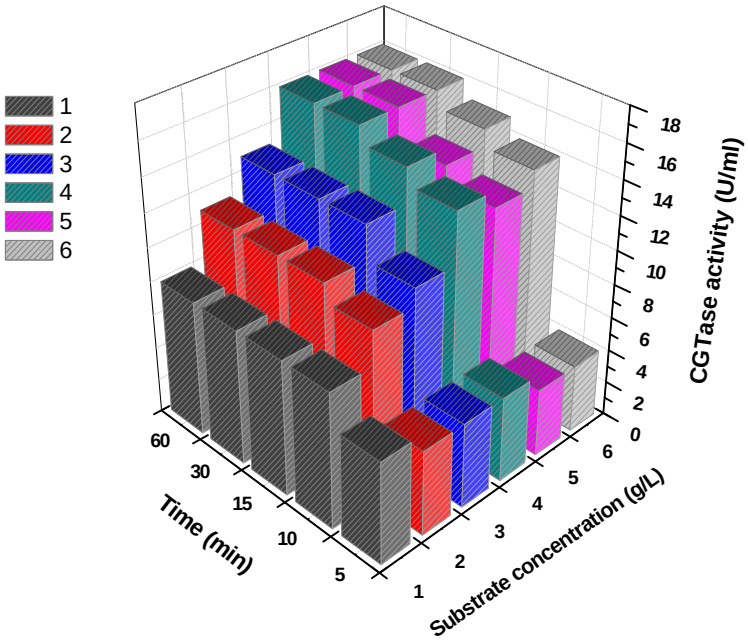

Figure 2: Effect of starch concentration and incubation time on CGTase activity

The observed saturated substrate concentration is less when compared to the reports of Pishtiyski *et al*., 2008. They have shown that the CGTase from the *Bacillus megaterium* having the maximum reaction substrate concentration of 10g/L. At higher concentration it showed negative impact on the enzyme activity.

Effect of pH on CGTase activity

In order to study the effect of pH on CGTase activity obtained from mutated *Bacillus* sp TPR71HNA6 the enzyme activity was measured at varying pH values ranging from 4.0 to 11.0 under standard assay conditions. The optimum pH of the purified CGTase was determined as pH 6.0 (Fig. 3). These results are in accordance with the literature reports. Sian *et al*., (2005) reported that CGTase obtained from the *Bacillus* sp G1 shows optimum pH at 6.0. Similarly the enzyme obtained from the *B. stearothermophilus* also shows the optimum activity at pH 6.0 (Chung *et al*., 1998).

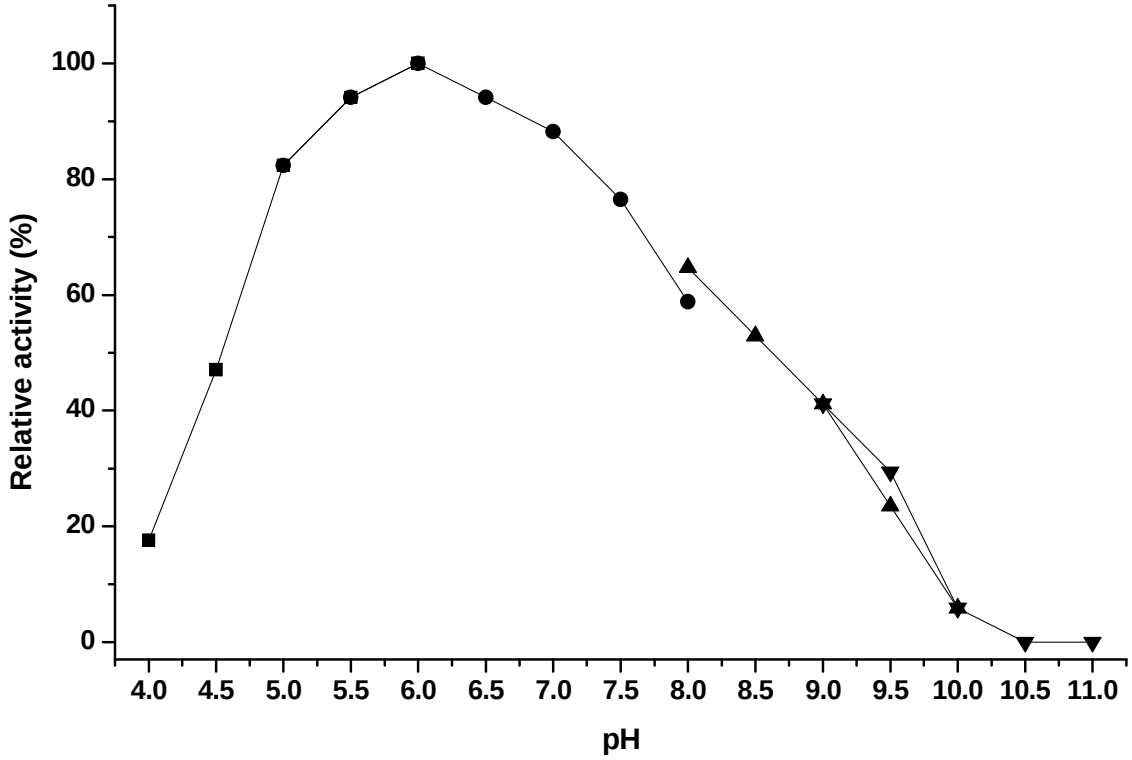

Figure 3: Effect of pH on purified CGTase activity

The obtained enzyme shows 90% activity at pH 5.5 and 6.5. It has 20% activity at pH 4.0 indicating that it has good pH stability. The complete inhibition of the enzyme was observed at pH 10.0. The purified CGTase showed good activity between pH 5.0 to 7.5 indicating that CGTase from *Bacillus* sp requires a near-neutral pH range to perform its reaction. This suggested that the extreme pH values were not suitable for the enzyme to carry out cyclization reactions. Most of the reported CGTase exhibited optimum pH ranging from 5.0 to 8.0 (Bovetto *et al*., 1992; Sohn *et al*., 1997;Tachibana *et al*., 1999) but the enzyme from *Brevibacterium* sp. no. 9605 possessed a higher optimum pH value at 10.0 (Mori *et al.,* 1994).

Effect of Temperature on CGTase activity

To study the effect of temperature on the purified CGTase, the activity of the purified enzyme was measured at different temperatures ranging from at pH 6.0 by the standard assay method. The optimum temperature for CGTase from mutated *Bacillus* sp TPR71HNA6 was 60^{o}C (Fig. 4). Above and below this temperature affects the enzyme activity. The loss of enzyme activity is high at higher temperature compared to the lower side.

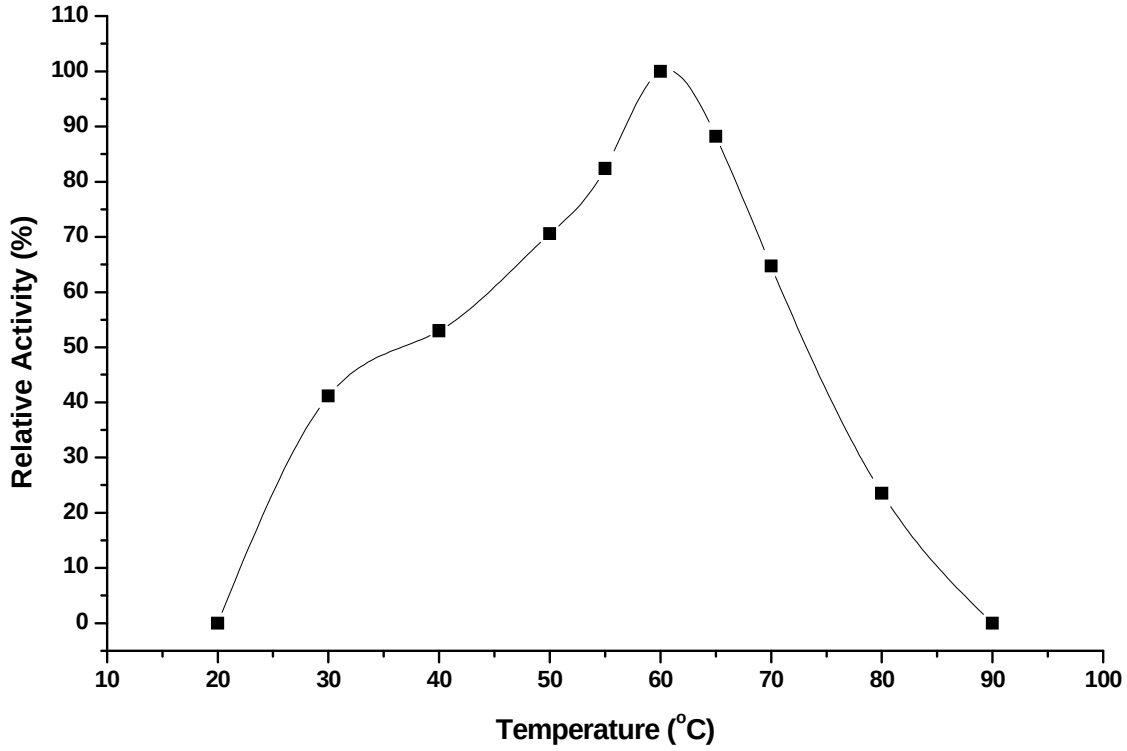

Figure 4: Effect of Temperature on purified CGTase activity

The obtained optimum temperature is in good agreement with the other reports. CGTase from the *Bacillus* sp G1 (Sian *et al*., 2005), *B. autolyticus* 11149 (Tomita *et al*., 1992), *B. stearothermophilus, Bacillus megaterium* (Pishtiyski *et al*., 2008) and *B. circulans* E 192 (Bovetto *et al*., 1992) also discovered 60^{o}C as the optimum temperature.

Determination of optimum pH and temperature by means of RSM

In order to understand the imperative role of pH and temperature on enzyme catalysis, optimum concentrations of variables for maximum CGTase activity were determined following the response surface methodology. Table 2 depicts the central composite design (CCD) experimental layout of the variables in both coded and natural units along with experimental results. The activity varied from 11 to 18U/ml indicating the influence of selected parameters levels role on enzyme activity. The data was analysed using Design expert software. A ±3 % variation in enzyme activity values between experimental and software predicted was noticed (Table 2) indicating the accuracy of experimentation. The coefficient of determination (R^2) was calculated as 0.9671 indicating that the statistical model can explain 96.71% of variability in the response (Fig. 5). The observed value of R^2 (0.9260) suggested a higher significance of the model.

Table 2: Central composite design along with the observed and predicted CGTase activity.

	pH (P)		Temperature (T)		CGTase activity		
S. No	**Coded**	**Real**	**Coded**	**Real**	**Observed**	**Predicted**	**Error**
1	-1.00	5.5	-1.00	55.0	13.00	12.46	0.53
2	-1.00	5.5	1.00	65.0	11.00	11.07	-0.07
3	1.00	6.5	-1.00	55.0	12.00	11.92	0.07
4	1.00	6.5	1.00	65.0	13.00	13.53	-0.53
5	-1.41	5.3	0.00	60.0	11.00	11.32	-0.32
6	1.41	6.7	0.00	60.0	13.00	12.67	0.32
7	0.00	6.0	-1.41	53.0	12.00	12.42	-0.42
8	0.00	6.0	1.41	67.0	13.00	12.57	0.42
9	0.00	6.0	0.00	60.0	17.00	17.50	-0.50
10	0.00	6.0	0.00	60.0	18.00	17.50	0.50

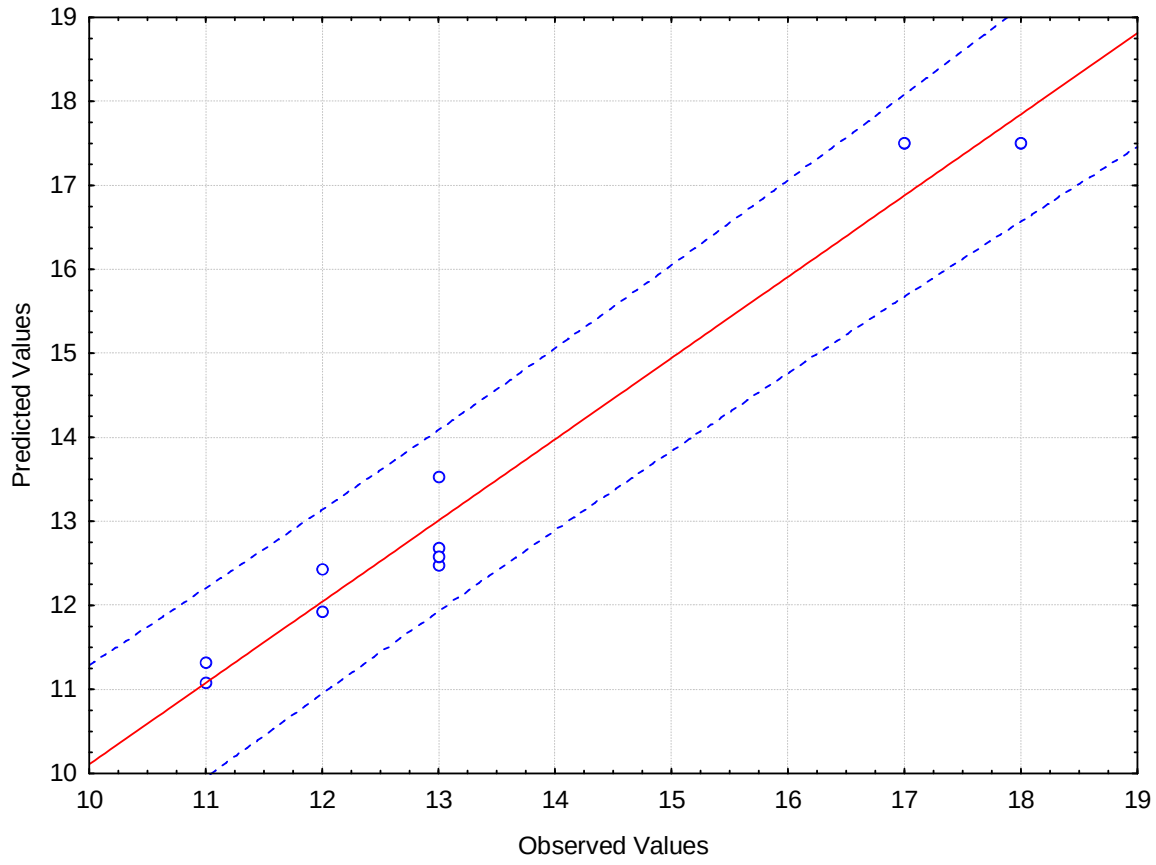

Figure 5: Correlation graph between the observed and predicted CGTase activity

To understand an empirical relationship between the CGTase activity (Y) and test variables (in coded units) as well as to perform the analysis of variance (ANOVA), the data was analyzed using second order polynomial model. In the

present study the following equation 1 was used.

$Y = 17.5000 + 0.95711*P + 0.1035*T - 2.7500 * P^2 - 2.5000 * T^2 + 0.7500 *P*T$ -------- (1)

where Y is response (CGTase activity).

Table 3 and 4 denotes the significance of each coefficient in terms of Student's *t*-test and p-values. It was observed that linear terms and interaction terms are insignificant. The quadratic term of pH has the highest magnitude and lowest p-value (-5.5, p= 0.000787) followed by quadratic term of temperature (-5.0, p=0.00113). Both pH and temperature have the highest effect at their quadratic term than the linear terms. It indicates that a little variation of these factors causes significant effect on the enzyme activity.

Table 3: Effects and regression coefficients

	Effect	**Coefficients**	**t-value**	**p-value**
Mean/ Interaction.	17.50000	17.50000	38.57529	0.000003
P	0.95711	0.47855	2.10975	0.102519
T	0.10355	0.05178	0.22826	0.830636
P*P	-5.50000	-2.75000	-9.16463	0.000787
T*T	-5.00000	-2.50000	-8.33148	0.001134
P*T	1.50000	0.75000	2.33802	0.079551

Table 4: Analysis of variance (ANOVA)

	SS	**df**	**MS**	**F-Value**	**p-Value**
P	1.83211	1	1.83211	4.45106	0.102519
T	0.02145	1	0.02145	0.05210	0.830636
P*P	34.57143	1	34.57143	83.99040	0.000787
T*T	28.57143	1	28.57143	69.41356	0.001134
P*T	2.25000	1	2.25000	5.46632	0.079551
Error	1.64645	4	0.41161		
Total SS	50.10000	9			

SS= Sum of Squares; MS = Mean square error; df= degree of freedom

The regression model data was represented in the form of 2D and 3D surface and contour plots. These graphs were used to predict the CGTase activity at different factor values of the test variables and to identify the major interactions between the test variables based on the circular or elliptical nature of contour. Each contour curve represents an infinite number of combinations of two test variables. The yield value for different concentration of the variables was predicted from the respective surface and contour plots. In the present study, the

generated contour is elliptical in nature indicating that these two factors are independent of each other suggesting there is no interaction between pH and temperature (Fig. 6). pH has a very narrow range to obtain the optimum conditions

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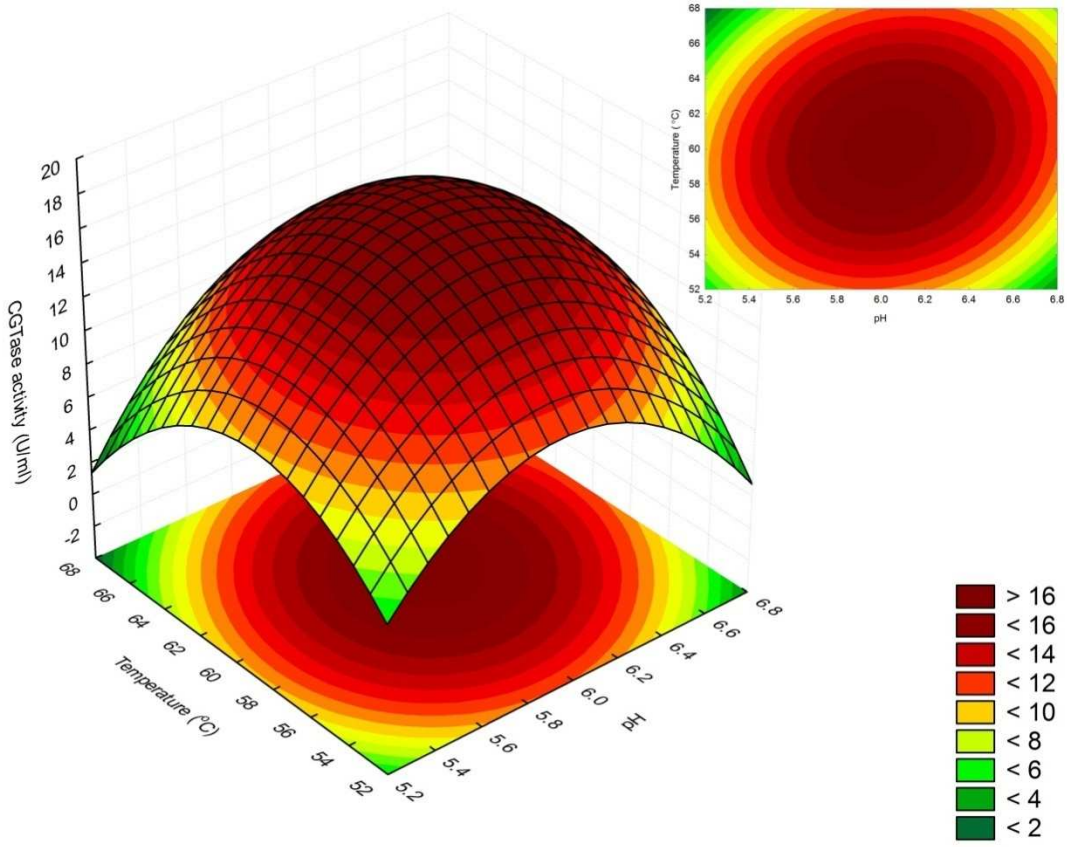

Figure 6: Interaction influence of pH and temperature on CGTase activity; insert: contour plot.

The optimal values of the pH and temperature obtained in coded values (X1 = 0.09 and X2 =0.23) were initially converted to real values. It was observed that the required optimized conditions include pH at 6.04, temperature 60.12^{o}C. In these conditions the predicted CGTase activity was 18U/ml. After conducting the experiments again it was noticed that 18U/ ml CGTase activity was obtained, indicating the proposed model has a good agreement with the observed values and also represents the accuracy of the experimentation.

pH and Thermal stability studies

The temperature effect studies and RSM studies revealed that the purified CGTase activity was decreased with increase of incubation temperature beyond 60°C. Based on the above data thermal stability study was performed by incubating the enzyme at 60 to 80°C with increase of 5°C up to 210 min. At various time intervals enzyme sample was withdrawn from the incubator and cooled to room temperature, enzyme activity was measured at room temperature. The remaining activity was compared against untreated enzyme activity and the same was represented as residual activity. Fig. 7

depicts the thermo stability study profile of CGTase obtained from the mutated *Bacillus* sp TPR71HNA6. The enzyme showed the good stability at 60°C, up to 90 min. After 60 min incubation the enzyme activity was decreased. The purified enzyme has good thermo stability, 50% of activity was retained at 210 min. In fact loss of activity was not observed at 60°C in the study.

The pH stability studies were performed by incubating the enzyme at various pH solutions for a longer time (up to 120 min) followed by measuring its activity at pH 6.0. The remaining activity was expressed as a residual activity. Fig. 8 shows the pH stability of the enzyme at various pH environments. It was observed that purified enzyme showed greater stability for longer time incubation at pH 6.0. The purified CGTase retained 50% of activity at 120 min incubation indicating that the obtained enzyme has a good pH tolerance. Above and below this pH the stability was low (Fig. 8). The obtained pH stability was higher when compared to other enzymes (Sian *et al*., 2005; Pishtiyski *et al*., 2008).

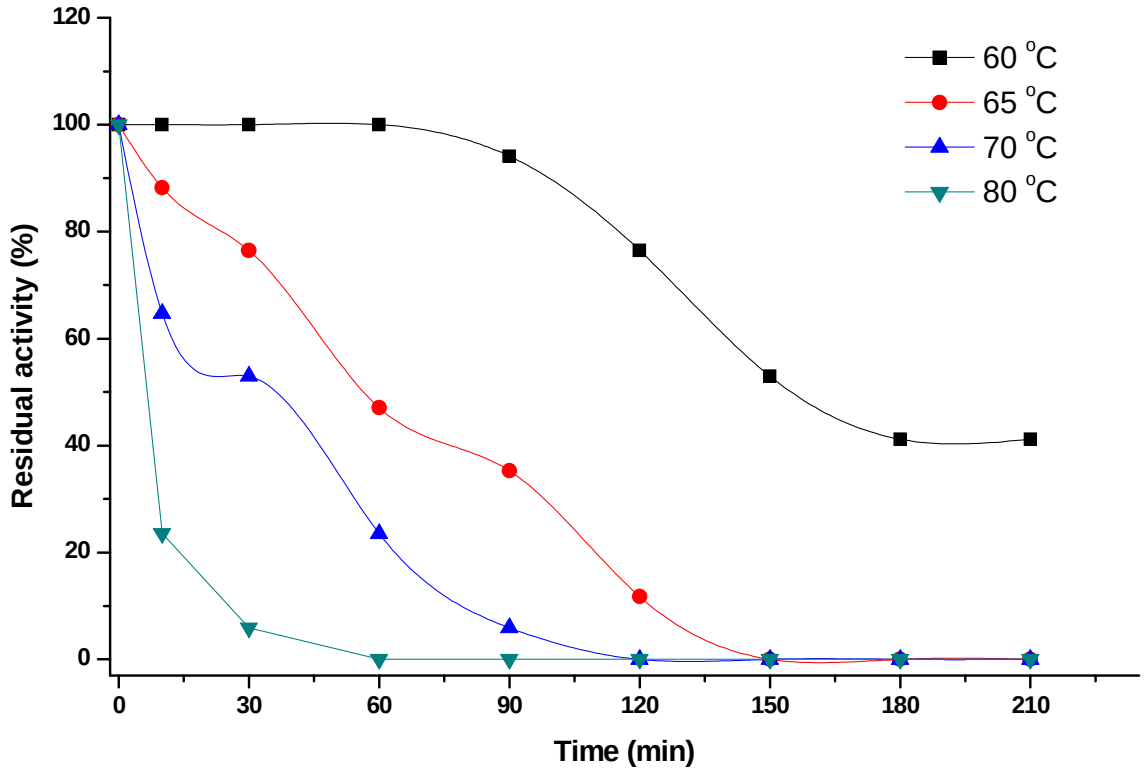

Figure 7: Thermostability studies of CGTase obtained from mutated *Bacillus* sp TPR71HNA6.

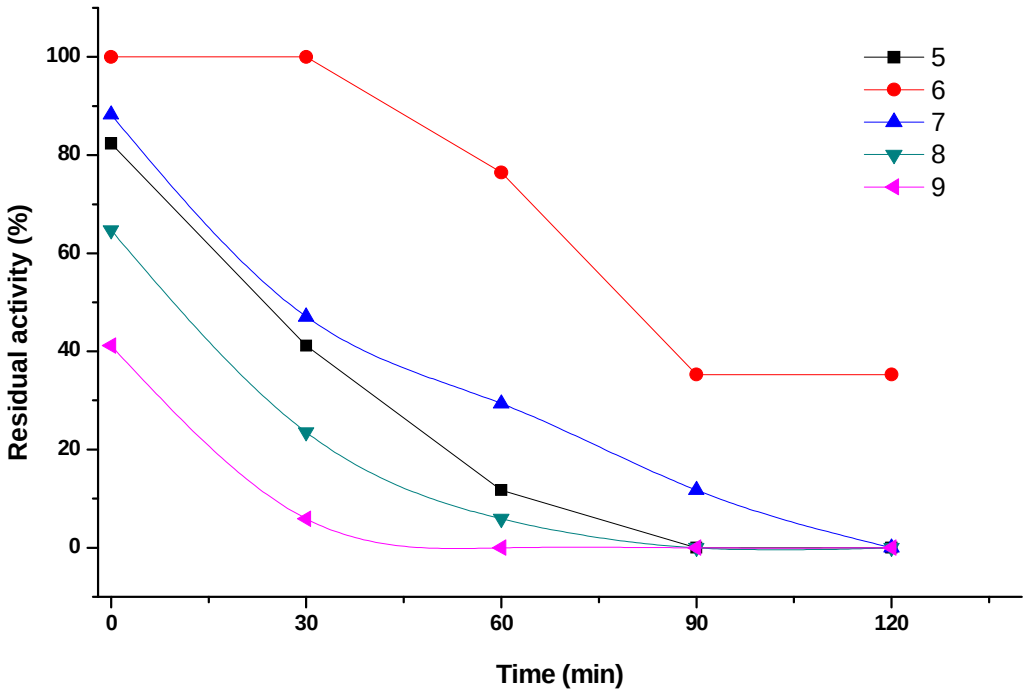

Figure 8: pH stability studies of CGTase obtained from mutated *Bacillus* sp TPR71HNA6

Effect of metal ions on CGTase activity

The effect of various metal ions and reagents on the purified CGTase obtained from the mutated *Bacillus* sp TPR71HNA6 is summarized in table 5.

Table 5: Influence of various metals and reagents on CGTase obtained from the mutated *Bacillus* sp TPR71HNA6.

Metal ions & Reagents	**CGTase Relative activity (U/ml)**	
	1mM	**2mM**
Control	100.00	100.00
$BaCl_2$	22.22	0.00
$CuCl_2$	38.89	0.00
$FeCl_3$	33.33	22.23
$MgSO_4$	50.00	44.45
$MnSO_4$	66.67	44.45
$ZnSO_4$	61.12	50.00

NaCl	77.78	66.67
$CaCl_2$	111.12	111.12
KCl	88.89	83.34
$CoCl_2$	0.00	0.00
$HgCl_2$	94.45	94.45
$AgNO_3$	94.45	72.23
$PbCl_2$	100.00	100.00
EDTA	50.00	33.34
SDS	66.67	44.45

The metal ions such as Ba^{+2}, Cu^{+2}, Fe^{+2}, Mg^{+2}, Mn^{+2}, Zn^{+2}, Na^{+}, K^{+}, Co^{+2}, showed the negative influence on the enzyme activity at higher and lower concentrations. Co^{+2} showed the higher inhibition activity on the isolated enzyme. The enhanced activity with Ca^{+2} was noticed while Pb^{+2} ions unaltered the enzyme activity. Whereas Hg^{+2} and Ag^{+2} have mild inhibitory effect on the CGTase activity obtained from *Bacillus* sp. The inhibitory effect of metal ions could be due to the oxidation of amino acid residues essential for the cyclization reaction. The reagents such as SDS and EDTA showed the inhibitory effect on the enzyme activity.

It is reported CGTase from *Brevibacterium* sp. no. 9605, *Bacillus agaradhaerens* (Pongsawasdi and Yagisawa, 1988), *Bacillus* sp. AL-6 (Fujita *et al*., 1990), *Bacillus halophilus* (Abelian *et al*., 1995), and *B. firmus* (Yim *et al*., 1997) had a significant inhibitory effect with Cu^{+2} and Zn^{+2} ions. However, it is interesting to find out that Fe^{+2} effects are contradicting to the literature reports. With CGTase from *Brevibacterium* sp., and *Bacillus* sp. G1 (Sian *et al*., 2005) it was observed that $FeCl_2$ enhanced the enzyme activity where as it is inhibiting the enzyme obtained from the *Bacillus* sp. Previous studies showed that CGTase from *Brevibacterium* sp. no. 9605 (Mori *et al*., 1994), *B. autolyticus* 11149 (Tomita *et al*., 1992) and *Paenibacillus* sp. F8 (Larsen *et al*., 1998) were very stable in their activity in the presence of Ca^{+2}. Thus, it is worth to note that CGTase from *Bacillus* sp. G1 (Sian *et al*., 2005) showed similar relative activity (11%) with *Bacillus* sp. under the study of Ca^{+2} effect. However, Abelian *et al*., 1995 observed that Ca^{+2} had no stabilizing effect on CGTase from *Bacillus halophilus*.

Estimation of kinetic parameters

Determination of V_{max} and K_m values

To determine kinetic parameters, the enzyme activity was measured in the presence of varying starch concentrations ranging from 1 to 6g/L. The obtained data was analyzed by non-linear regression

method by using Graph Pad prism V4.0. The non-linear regression of the data revealed that K_m (affinity constant) is 0.866 ± 0.26 µmol and V_{max} (maximal velocity) is 16.1 ± 1.1U/ml/min (Fig. 9).

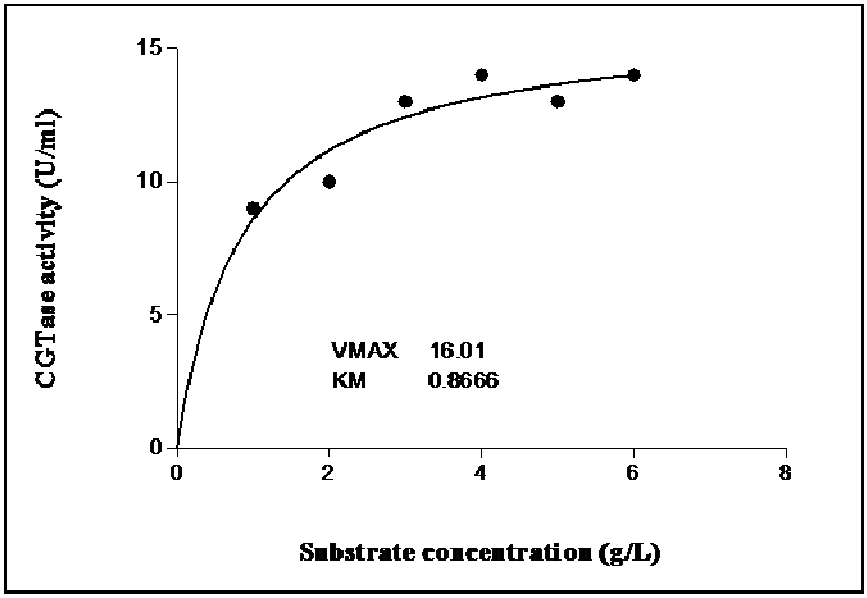

Figure 9: The Michaelis-Menten plot to determine the K_m and V_{max} for CGTase from mutated *Bacillus* sp TPR71HNA6.

The results indicated that *Bacillus* sp has a relatively high affinity for the substrate. These results also showed that CGTase produced by *Bacillus* sp was comparatively more active than the other reported CGTases, since it needed a much lower concentration of soluble starch to achieve V_{max}. Different K_m values have been reported for various CGTases, namely CGTase from *Bacillus megaterium*, 3.4g/L (Pishtiyski *et al*., 2008) *B. circulans* E192 5.7mg/ml (Bovetto *et al*., 1992); *B. firmus* 1.21mg/ml (Gawande *et al*., 1999); *K. pneumoniae* AS- 22 1.35 mg/ml (Gawande *et al*., 1998); *Bacillus agaradhaerens* 21.2mg/ml (Martins and Hatti-Kaul, 2002), with soluble starch as the substrate; while K_m value for CGTase from *Bacillus* sp. was 33.9mg/ml with cassava starch as the substrate (Wang *et al*., 1995).

Determination of activation energy (E_a)

The activation energy (E_a) of CGTase from mutated *Bacillus* sp TPR71HNA6 was calculated by using the Arrhenius plot by taking the value of Ln *v* against the reciprocal of absolute temperature (Fig. 10). As seen, the temperature dependence of the inactivation constant followed the Arrhenius equation. The energy of activation E_a calculated from the slope of the Arrhenius plot was

24.20kJmol^{-1}. The lower activation is desirable for the enzyme studies.

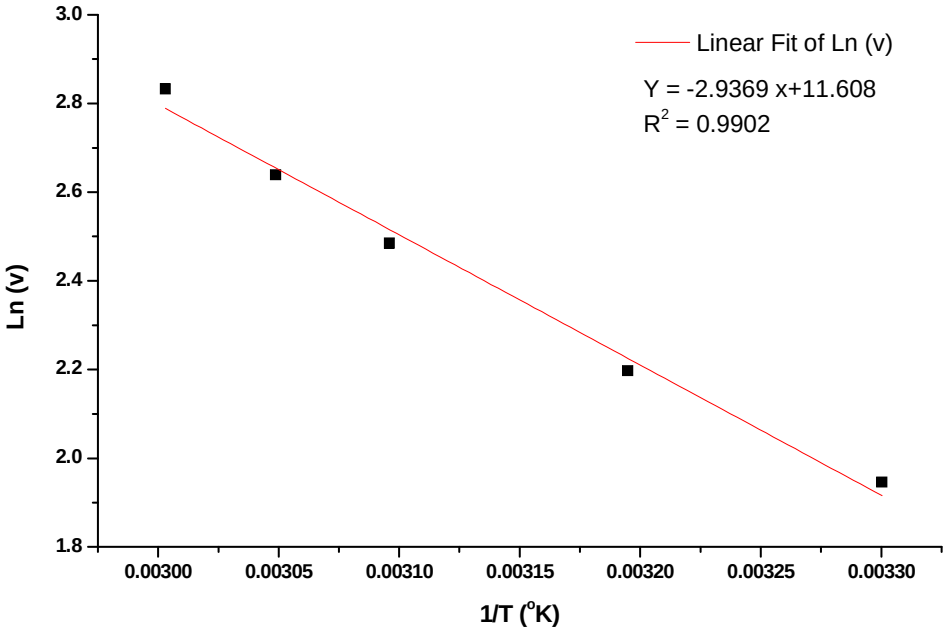

Figure 10: Arrhenius plot of the CGTase obtained from mutated *Bacillus* sp TPR71HNA6.

CONCLUSION

The present study explains the purification of CGTase produced by mutated *Bacillus* sp TPR71HNA6. Results of the present study indicated that the molecular weight was 75KDa and after purification the specific activity of the CGTase was increased from 97.51U/mg to 307.69U/mg and finally 30% was recovered with 3.15% purification fold.

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