Int J Pharm Bio Sci 2013 Jan; 4(1): (B) 515 - 523

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

PRODUCTION OF CYCLODEXTRIN GLYCOSYL TRANSFERASE FROM ALKALIPHILIC *Paenibacillus sp* L55 MCM B - 1034 ISOLATED FROM ALKALINE LONAR LAKE, INDIA

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ABSTRACT

Cyclodextrin glycosyl transferase (CGTase; EC 2.4.1.19) is an enzyme which converts starch into cyclodextrins (CDs). Cyclodextrins are closed-ring structures in which six or more glucose units are joined by α-1, 4 glucosidic bonds. Cyclodextrins have applications in food, pharmaceutical and cosmetic industries. This paper reports the exploration of aerobic alkaliphilic bacteria from alkaline soda lake of Lonar, India for CGTase production. *Paenibacillus* sp L55 isolated from Lonar lake was used for production of CGTase. Three medium components Starch, Peptone and Yeast extract and initial pH of medium were optimised by Taguchi Design of Experiments (DOE). The contribution of starch in CGTase production was the highest while pH showed the least contribution in CGTase production. The optimised medium was used for CGTase production and the yield of CGTase was increased from 3 U/ml to 5.1 U/ml.

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Key words: **Cyclodextrin glycosyl transferase, Lonar lake, *Paenibacillus* sp L55, Taguchi DOE, alkaliphilic bacteria**

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INTRODUCTION

The alpha amylase family is an extensively studied family which comprises of several enzymes. Cyclodextrin glycosyl transferase (CGTase, EC 2.4.1.19) is a member of this family which produces cyclodextrins from starch. CGTases are known to catalyze four different transferase reactions: cyclisation, coupling, disproportionation, and hydrolysis. Three major types of Cyclodextrins (CD) are produced by CGTases depending on number of glucose units, viz. α – CD, β- CD and γ- CD .Cyclodextrins are oligosaccharides which contain glucose molecules joined by α-1, 4 glycosidic linkage. CD's are used in analytical chemistry, agriculture, biotechnology, cosmetics, pharmacy and food[1]. Cyclodextrins are used as drug carriers and tabletting vehicles and are used to reduce the bitter or irritant taste and bad smell of drugs [2, 3]. Cyclodextrin can improve the stability of active pharmaceutical ingredients and increase the shelf life of drugs. It increases stability and solubility of cosmetic active ingredients and provides a better control over the release of fragrances. It is evident from the above mentioned applications that CGTase enzyme is industrially a very important enzyme because of its application in production of CD's. In nature, CGTase is produced by *Bacillus* sp., *Klebsiella*, *Pseudomonas*, *Brevibacterium*, *Thermoanaerobacterium, Corynebacterium, Micrococcus* and *Clostridium.* Recently scientists have started exploring extremophiles for CGTases with novel properties. CGTase from alkaliphilic *Bacillus* sp. lead to mass production of α – CD, β- CD and γ- CD[4]. Since then, it is known that alkaliphiles are candidate organisms for CGTase production. Menocci *et.al.* (2008) have optimised CGTase production from *Bacillus* sp isolated from Brazilian soil using conventional method[5]. Rosso *et. al* (2002) have used a combination of conventional and statistical methods to optimise CGTase production from *Bacillus circulans* DF 9R[6]. Bonilha *et. al* (2006) have also used conventional optimisation techniques for optimisation of CGTase production by *Bacillus licheniformis*[7]. Conventional optimization procedures involve altering of one parameter at a time keeping all other parameters constant, which enables one to assess the impact of those particular parameters on the process performance. These procedures are time consuming, cumbersome, require more experimental data sets and cannot provide information about the mutual interactions of the parameters.[8] Alternative to conventional optimization procedures, Design of Experiments (DOE) and statistical tools help to gain more information about the optimization conditions in a few trials[9]. Statistical experimental design methods provide a systematic and efficient plan for bioprocess optimization considering the interactive effects among the control factors. Many control factors can be simultaneously studied and optimized by statistical experimental designs [10, 11.] The most commonly used statistical designs are Plackett Burman method, Response surface methodology, Central composite Design and factorial design. Noi *et.al* (2008) has optimised CGTase production from *Bacillus* sp using response surface methodology[12]. Gawande and Patkar (2001) have used factorial method to optimise CGTase production from *Klebsiella pneumoniae*[13]. Another method used for statistical optimisation is the Taguchi Design of Experiments. The Taguchi approach of DOE originally developed by Dr. Genichi Taguchi has been the popular method and process improvement tool in the hands of the engineering and scientific professionals. The basic principle of this method serves as screening filters which examine the effects of many process variables and identify those factors which have major effects on process using a few experiments[14]. Taguchi method of DOE involves establishment of large number of experimental situations described as Orthogonal Arrays (OA) to reduce experimental errors and to enhance their efficiency and reproducibility of the laboratory experiments[9]. This method is now being used in biotechnology for optimisation of enzymes

like alkaline protease, lipase, polygalacturonase production, exopolymer production, single cell protein production from methanol, butanediol production and also optimisation of parameters involving scale up[15, 16,17,18,19]. The advantages of using the Taguchi method are that many more factors can be screened and optimized simultaneously and much quantitative information can be extracted by only a few experimental trials. Therefore, these methods have been extensively applied in parameter optimization and process control[20] .In this paper we have used *Paenibacillus* sp L55 MCM B-1034 isolated from alkaline Lonar lake, India for production of CGTase, application of Taguchi methodology for optimisation of CGTase production and production of cyclodextrins from starch using CGTase.

MATERIALS AND METHODS

1. Screening of alkaliphilic bacteria from Lonar Lake for production of CGTase

Aerobic alkaliphilic bacteria previously isolated and identified from Lonar lake were screened for starch hydrolysis using Nutrient agar containing 1 % starch[21]. Organisms showing starch hydrolytic activity were further screened for CGTase production on Phenolphthalein methyl orange agar medium containing 1.0% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4$-$7H_2O$, 1% Na_2CO_3, 1.5% agar, 0.03% phenolphthalein and 0.01 % methyl orange . The organisms showing yellow halo on phenolphthalein methyl orange agar medium were selected for production of CGTase which was confirmed by CGTase assay using phenolphthalein reagent assay (PHP assay) as described by Goel and Nene (1995)[22].

2. Micro-organism and growth condition

The alkaliphilic bacterium *Paenibacillus* sp L55 was previously isolated from Lonar Lake, India and identified by biochemical tests and 16S rRNA sequencing previously [21.] The strain was maintained on Nutrient medium containing (g/L) soluble potato starch 10, peptone 10, yeast extract 5, sodium chloride 5 and Agar 20. The pH was maintained as 10 by addition of sterile 10 % sodium carbonate after autoclaving. The organism was cultivated in 250 ml flasks containing 50 ml medium incubated in an orbital shaker at 150 rpm at 30° C for 24 hours.

3. Enzyme Assay

100 µl of crude enzyme extract was added to 1 ml of 1 % soluble potato starch in 0.005 M Tris Cl buffer pH 8.5 and incubated at 60^o C for 20 min. After incubation, this reaction mixture was cooled in ice. 4 ml of 1mM phenolphthalein reagent was added to the tubes and the absorbance measured immediately at 550 nm. The amount of β-CD produced is estimated from standard graph of 50 – 200 µg /ml of standard β-CD (Sigma-Aldrich) against decrease in absorbance[22]. One unit of CGTase activity is defined as an amount of enzyme required to produce 1 µg of β-CD /ml/min.

4. Optimisation of production using Taguchi Design Of Experiments (DOE)

The components of production medium viz. starch, peptone, yeast extract and pH at three different levels were selected for optimisation using Taguchi DOE. L9 orthogonal array experimental design as in Table 1 was used for optimisation. Ten percent of 18 hour old inoculum with initial cell density of 10^9 Colony forming units ml^{-1} was added to 50 ml of medium with ingredients at various levels as described in Table 1.

Table 1
Parameters optimised for CGTase production by Paenibacillus sp L55 at various levels used in L 9 orthogonal array design

Parameter	Level 1	Level 2	Level 3
Starch (%)	0.5	1	5
Peptone (%)	0.5	1	2.5
Meat Extract (%)	0.1	0.3	1
pH	8	9	10

The flasks were incubated at 30°C for 24 h at 150 rpm. After 24 h, the broth was centrifuged at 10,000 rpm at 4°C for 20 min. The cell free supernatant was used as crude enzyme and the enzyme activity was estimated as described above. All experiments were performed in duplicates, each sample was analysed in triplicates. The experimental results were analyzed to extract independently the main effects of the factors; the analysis of variance technique was then applied to determine statistically significant factors. The controlling factors were identified, with the magnitude of effects qualified and the statistically significant effects determined. Accordingly, the optimal conditions were determined by combining the levels of factors that had the highest main effect value. A validation experiment is performed under the predicted optimised condition to ascertain the enzyme activity in optimised conditions.

RESULTS

1. Screening of alkaliphilic bacteria for CGTase production

15 alkaliphilic Lonar lake isolates showed starch hydrolytic activity and 6 were positive for CGTase production on Phenolphthalein methyl orange medium. The results of confirmation of CGTase production by PHP assay are presented in Table 2.

Table 2
Screening of alkaliphilic bacteria from Lonar Lake for CGTase production

No.	MCM number	Name of organism	Starch Hydrolysis (Amylase)	PHP Assay (CGTase)
1	MCM B-1001	*Bacillus cereus*	+	--
2	MCM B-1016	*B. firmus*	+	**+**
3	MCM B-1036	*B. flexus*	+	--
4	MCM B-1044	*B. fusiformis*	+	**+**
5	MCM B-1010	*B. licheniformis*	+	**+**
6	MCM B-1035	*B. benzoevorans*	+	--
7	MCM B-1038	*B. cohnii*	+	--
8	MCM B-1041	*Alkalibacillus haloalkaliphilus*	+	--
9	MCM B-1034	*Paenibacillus sp L 55*	+	**+**
10	MCM B-1018	*Vagococcus carniphilus*	+	--
11	MCM B-1027	*Halomonas campisalis*	+	--
12	MCM B-1025	Lake Bogoria isolate 25 B 1	+	**+**
13	MCM B-1046	*Alkalimonas delamerensis*	+	--
14	MCM B-1021	*Exiguobacterium aurantiacum*	+	**+**
15	MCM B-1006	*Arthrobacter mysorens*	+	-

Key +: produces enzyme, - : negative for enzyme production

The six isolates showing positive CGTase activity are *Bacillus firmus, Bacillus fusiformis, Bacillus licheniformis, Paenibacillus sp L55, Exiguobacterium aurantiacum* and Lake Bogoria isolate 25 B1. To the best of our knowledge, this is the first report of alkaliphilic *Exiguobacterium aurantiacum* and Lake Bogoria isolate 25 B1 and *Paenibacillus sp L55* from Lonar lake for CGTase production. On the basis of enzyme activity, specific activity and amount of Cyclodextrin produced, *Paenibacillus sp L55* (MCM B-1034) was selected for CGTase production.

2. Optimisation of CGTase production by Paenibacillus sp L55 using Taguchi Design of experiments

CGTase was produced in shake flask fermentation with an average enzyme activity of 3 U/ml. The experimental design and CGTase activity obtained using Taguchi DOE is reported in Table 3. The enzyme activity varied from 3.6 U/ml to 5.5 U/ml.

Table 3
Taguchi Design of experiment by L9 Orthogonal Array for CGTase production by Paenibacillus sp L55.

Flask No	Factor 1 Starch	Factor 2 Peptone	Factor 3 Yeast Extract	Factor 4 pH	Enzyme Activity U/ml
1	1	1	1	1	5.15
2	1	2	2	2	4.4
3	1	3	3	3	5.25
4	2	1	2	3	5.00
5	2	2	3	1	5.05
6	**2**	**3**	**1**	**2**	**5.5**
7	3	1	3	2	4.2
8	3	2	1	3	3.65
9	3	3	2	1	4.2

The concentrations of tested factors 1, 2, 3 as well as values of factor 4 at levels 1, 2, and 3 are as illustrated in Table 1.

On the basis of analysis, the optimised medium components were as follows: (in g/L): starch 5, peptone 25 and yeast extract 1 and initial pH 9 with optimum enzyme activity of 5.5 U/ml (Flask no. 6). The validation experiments were done in triplicate to obtain the maximum yields of CGTase from *Paenibacillus* sp. The yield of CGTase obtained was 5.15 U/ml. This indicates the importance of parameter optimization for formation of any product and the role of various physicochemical parameters including medium composition and pH of the medium in microbial metabolism. Such factor-mediated regulation of microbial fermentation has been observed with many microbial species by Prakasham *et al*. (2007).

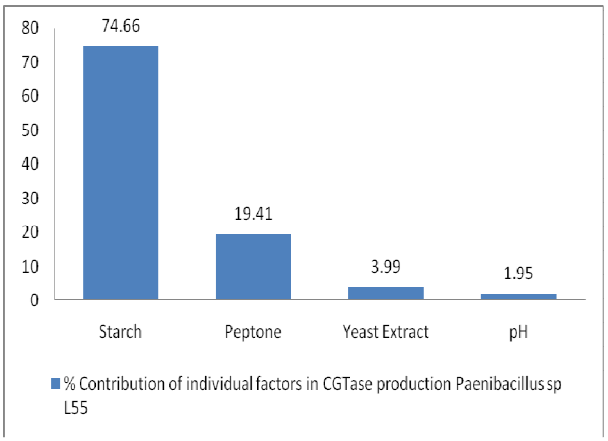
The difference between level 2 and level 1 (L2−L1) of each factor indicates the relative influence of the affect (Table 4). The larger the difference, the stronger is the influence.

Table 4
The average effect of parameters along with interactions at the assigned levels on CGTase production by Paenibacillus sp L 55.

Parameter	L1	L2	L3
Starch	13.836	14.284	12.059
Peptone	13.56	12.727	13.892
Yeast extract	13.43	13.104	13.645
pH	13.589	13.38	13.209

It can be seen from Table 4, that among the factors studied, starch showed stronger influence compared to other factors followed by peptone, yeast extract, and initial medium pH in the CGTase production. The medium designing and optimisation aims not only to support the growth of desired organism but also to facilitate enhanced production of the desired product. It is evident from Graph 1 that there is significant interaction between the parameters. The analysis revealed that starch (74.66 %) is the most significant parameter and essential nutrient for CGTase production. Amongst peptone and yeast extract, peptone was more significant than yeast extract and initial pH of medium was the least significant (1.95 %) (Graph 1).

Graph 1
Percentage Contribution of each factor in CGTase production by *Paenibacillus* sp L55



The percentage contribution of each parameter on CGTase production and significance has been determined by ANOVA. Based on F-ratio, starch is the most significant parameter for CGTase production. The validation experiments resulted in CGTase yield of 5.15 U/ml which is almost >1.5 times the yield obtained by unoptimised approach.

DISCUSSIONS

The ability of CGTase to convert starch into favoured industrial substance called cyclodextrin through cyclisation process is of great interest to researchers due to its enormous applications. CGTase production is highly dependent on the strain, medium composition and culture conditions. Alkaliphiles are known to be suitable candidate organisms for CGTase production[4]. The exploration of alkaliphiles from Lonar lake led to an interesting finding of *Exiguobacterium aurantiacum*, Lake Bogoria isolate 25 B1 and *Paenibacillus sp L55* from Lonar lake for CGTase production. Optimisation methodology is thus a very crucial part for any industrial fermentation for generation of maximum product with fewer trials. Taguchi approach is a promising method which has advantage of maximum experimental conditions with multiple parameters varied at the same time in fewer trials. Analysis of variance (ANOVA) is used to analyze the results of the OA experiment and to determine how much variation each factor has contributed. From the calculated ratios (*F*), it can be referred that all factors and interactions considered in the experimental design are statistically significant effective at 95% confidence limit, indicating that the variability of experimental data explained in terms of significant effects. By studying the main effects of each of the factors, the general trends of the influence of the factors towards the process of enzyme production can be characterized. The characteristics can be controlled such that a

lower or a higher value in a particular influencing factor produces the preferred result. Thus, the levels of factors to produce the best results can be predicted and the result can be confirmed using validation experiments. The data obtained in the trials show that CGTase production varied from 3.65 to 5.5 U/ml demonstrating the significance of parameters in CGTase production. The key nutrient showing maximum percentage of contribution to variance is starch.

Menocci *et. al.* (2008) have also reported that potato starch is the best and most significant carbon source for CGTase production using *Bacillus* sp. Bonilha *et.al.* (2006) have also reported maximum specific activity of CGTase in presence of starch. It has been reported that some starches may contain an inducer for CGTase production. The difference in CGTase activity obtained with different starches may be due to the differences in their physical structures and chemical properties .Noi .*et.al* (2008) have also reported significant contribution of sago starch for CGTase production using *Bacillus* sp. However Gawande and Patkar (2001) have reported the advantage of dextrin as carbon source over starch. Taguchi methodology was thus successfully employed to enhance the production of CGTase from *Paenibacillus* sp L55. CGTase production has been previously reported from organisms like *Paenibacillus azotofixans*, *Paenibacillus macerans* and *Paenibacillus* sp RB01 using various optimisation methodologies like response surface, central composite and Taguchi DOE but this is the first report of production of CGTase from alkaliphilic *Paenibacillus* sp L55 from soda lake of Lonar, India[23, 24, 25, 26].

CONCLUSIONS

Alkaliphiles from Lonar lake namely *Bacillus firmus, Bacillus fusiformis, Bacillus licheniformis, Paenibacillus sp L55, Exiguobacterium aurantiacum* and Lake Bogoria isolate 25 B1 were found to produce CGTase. Optimisation of production of CGTase by the alkaliphilic *Paenibacillus* sp. L55 MCM B – 1034 using Taguchi Design of experiments resulted in enhancement of enzyme activity from 3 U/ml to 5.15 U/ml with the highest contribution by starch as the substrate. *Paenibacillus sp L55* thus shows potential in production of cyclodextrins from starch.

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