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CYCLODEXTRIN GLYCOSYLTRANSFERASE PRODUCTION BY ALKALIPHILIC *BACILLUS SP.* ISOLATED FROM RICE CULTIVATED SOIL AND MEDIA OPTIMIZATION USING TAGUCHI METHOD

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Keywords:
Cyclodextrin glycosyltransferase (CGTase), alkaliphile, tapioca starch, factorial design.

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ABSTRACT: A Cyclodextrin glycosyltransferase (CGTase) producer was isolated from soil obtained from rice fields using alkaline Horikoshi II medium. The alkaliphile was characterized microscopically, biochemically and confirmed by 16S rRNA analysis as *Bacillus oshimensis*. CGTase production is dependent on the strain, medium composition and culture conditions; hence media optimization studies were carried out in shake flask using the statistical method. Tapioca starch was used as carbon source and a combination of yeast extract and peptone were used as nitrogen source in the media, along with $MgSO_4.7H_2O$, K_2HPO_4 and Na_2CO_3. A 3^3 factorial design using Taguchi method has been chosen to elucidate the combined effect of these variables. The parameters were optimized by changing three independent variables, with fixed concentration of mineral sources. The optimal media composition for CGTase production was found to be comprised of Tapioca starch 1%; peptone 0.5%; yeast extract 0.5% and 0.14% Magnesium Sulphate, 0.2% ammonium dihydrogen phosphate according to the factorial designing of the experiment and was used for further studies. The CGTase activity was found to be 4.5 U/ml after media optimization.

INTRODUCTION: Cyclodextrin glycosyl transferase (EC 2.4.1.9) is a unique enzyme capable of converting starch and related substrates into cyclomaltodextrins (CDs) [1]. CDs are cyclic oligosaccharides consisting of α-1, 4-linked 6, 7, or 8-glucopyranose units, usually referred to as α, β, or γ-CDs respectively.

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CDs possess a unique torus shape and the polar hydroxyl groups are oriented toward the outside, keeping the interior cavity relatively hydrophobic.

Therefore, CDs are soluble in water and the hydrophobic environment of the cavity enables them to form inclusion complexes with many organic and inorganic molecules, thereby changing the physical and chemical properties of the included compounds. This is the basis of broad applications in the agriculture, textile, food, cosmetic, and pharmaceutical industries as well as in bioconversions and separation processes [2, 3, 4].

There are several known alkaliphilic *Bacillus sp*. that are able to produce CGTase.

The bacterial CGTase is a multifunctional extracellular, inducible enzyme produced by a variety of bacteria such as *Bacillus halodurans* [5], *Bacillus firmus* [6], *Bacillus circulans* [7], *Bacillus stereothermophilus, Bacillus licheniformis* [8], *Bacillus alcalophilus* [9], *Amphibacillus sp.* NPST – 10 [10], *Bacillus lehensis* [11], *Bacillus sp.* A2-5a [12], *Paenibacillus pabuli* US 132 [13] and *Thermoanaerobacter spp.* P4 [14]. Rapid enzyme production can be achieved by manipulation of media composition and culture conditions [15, 16]. The present work carried out includes isolation of alkaline CGTase producer and optimization of media composition for better enzyme production and activity.

MATERIAL AND METHODS:

Screening and isolation of the strain: The alkaliphilic CGTase producer was isolated from rice cultivated soil in Pen (18°40'North, 73°05'East), Navi Mumbai, India. Soil (1 g) was suspended in 10 ml of sterile saline and was allowed to settle down. Supernatant (0.1 ml) was plated on alkaline Horikoshi (II) agar plate, pH 10.5 containing 1% soluble starch, 0.5 % peptone, 0.5% yeast extract, 0.1% KH_2PO_4, 0.02% $MgSO_4.7H_2O$, 2% agar, 0.03% phenolphthalein and 1% Na_2CO_3. The plates were incubated at 37°C for 24 h. Screening of CGTase producer was done according to Park *et al*. 1989 [17].

Formation of yellowish halo zones around the colonies was considered as an initial indication of CGTase activity. The bacterial colony with biggest halo zone was selected and streaked on Horikoshi (II) medium for several times until uniform colonies were formed.

Characterization and identification of Bacteria: The morphological characteristics of the isolate were examined on Horikoshi agar. The size, shape, presence of endospores and motility of the cells were determined. The biochemical characterization was done according to the method of Bergey's Manual of Determinative Bacteriology. 16S rRNA sequencing of the strain was carried out for further identification and classification by NCCS (National Centre of Cell Science, Pune).

Preparation of bacterial inoculums: The selected bacterial strain was grown in 50 ml of alkaline Horikoshi (II) medium (basal medium), pH 10.5 containing 1% soluble starch, 0.5 % peptone, 0.5% yeast extract, 0.1% KH_2PO_4 , 0.02% $MgSO_4.7H_2O$ and 1% Na_2CO_3. The culture was incubated at 37° C for 24 h on orbital shaker at 120rpm. The cells were harvested at 5,000 rpm for 15 min, washed with normal saline (0.85% w/v NaCl) and were then suspended in normal saline to give an optical density reading of 0.05 at 660 nm which was utilized as the inoculum.

CGTase assay: CGTase activity was determined using phenolphthalein assay method [18] with slight modifications. Reaction mixture containing 1% soluble starch in 50mM Tris HCl (pH 10), and 1ml of crude enzyme was incubated at 37°C for 15 min. The reaction was stopped by incubating this reaction mixture in boiling water bath for 3 min. To this 4 ml of 0.04 mM phenolphthalein dissolved in 125 mM $Na_2CO_3.NaHCO_3$ buffer solution was added. The absorbance was measured at 550 nm. The amount of β-cyclodextrin produced was estimated from the standard graph of 0-500 µg/mL β-CD concentration against absorbance. One unit (U) of enzyme activity was defined as the amount of enzyme that produced 1µmol of β CD per minute under standard conditions [19].

Growth Kinetics: The growth kinetics for the isolated bacterial culture was studied and compared by inoculating the alkaline Horikoshi (II) medium (basal medium), pH 10.5, containing 1% soluble starch, 0.5 % peptone, 0.5% yeast extract, 0.1% KH_2PO_4, 0.02% $MgSO_4.7H_2O$, 1% Na_2CO_3 and the optimized alkaline media containing 1% tapioca starch, 0.5 % peptone, 0.5% yeast extract, 0.14% Magnesium Sulphate, 0.2% ammonium dihydrogen phosphate and 1% Na_2CO_3.

Two percent (v/v) bacterial culture was inoculated in 50 ml of basal and optimized medium in a 250 ml shake flask. The fermentation media were incubated at 37°C on an incubator shaker at 120 rpm. Aliquots (2 ml) were withdrawn every 2 h for 96 h. The optical density was determined at 660 nm and the supernatant obtained after centrifugation at 10,000 rpm for 15 min was assayed for CGTase activity.

Effect of initial pH: The effect of initial pH on CGTase production was studied by varying Na_2CO_3 concentrations (0-1% w/v) in basal fermentation media. The concentrations correspond to initial pH of 6, 7, 8, 9 and 10. Two percent of the bacterial inoculum was inoculated into Horikoshi (II) medium in a 250 ml shake flask. The medium was incubated at 37^{o}C for 24 h at 120 rpm. The cells were harvested and the supernatant was analyzed for CGTase activity.

Effect of carbon and nitrogen source: Effect of carbon sources on growth and CGTase production was investigated by substituting soluble starch (1%) in standard Horikoshi II medium with different types of carbon sources such as glucose, lactose, maltose, dextrin, potato starch, tapioca starch, corn starch, wheat starch, rice starch, sorbitol and mannitol. Similarly effect of nitrogen sources was evaluated by replacing peptone and yeast extract with different types of organic and inorganic nitrogen sources such as peptone, yeast, malt extract, beef extract, casein, casein acid hydrolysate, urea, sodium nitrate, ammonium chloride, ammonium sulphate, ammonium nitrate, potassium nitrate and tryptone. The most suitable carbon and nitrogen source for CGTase production were chosen and incorporated in basal media for further studies.

Effect of Phosphorus source: Different types of phosphorus source such as potassium dihydrogen phosphate, potassium hydrogen phosphate, sodium dihydrogen phosphate, sodium hydrogen phosphate, ammonium dihydrogen phosphate (0-0.2%) were studied. The most suitable phosphorus source in production of CGTase was chosen for the subsequent experiments.

Effect of concentration of Magnesium sulphate: Magnesium sulphate facilitates the growth of *Bacillus species*, hence variations in concentrations of magnesium sulphate (0 - 0.2%) were studied. After 24 h of incubation the supernatant was analyzed for CGTase activity.

Design of Experiment by Taguchi method: The fractional factorial design of experiments was carried out using the Taguchi method. The Taguchi method utilizes orthogonal array design to study a large number of parameters with a small number of experiments. It significantly reduces the number of experimental configurations to be studied without affecting the quality of results. In this study, parameters considered in the optimization process were: Tapioca starch (A), Peptone (B) and Yeast Extract (C) and each parameter consists with three levels (values) as shown in **(Table 1)**. L_9 orthogonal arrays improved by Taguchi method was chosen to determine the experimental design.

TABLE 1: PARAMETERS AND LEVELS USED IN FACTORIAL DESIGN

Level	Factor		
	Tapioca Starch (g%) (A)	Peptone (g%) (B)	Yeast Extract (g%) (C)
1	1	0.5	0.5
2	2	1.0	1.0
3	3	1.5	1.5

The quality of results was measured by the signal to noise ratio (S/N) ratio with the target values of 'larger is better', since the purpose of this study was to have high yield of CGTase. In this study, interactions among variables were not considered and focus was placed on the main effects of three parameters.

Statistical Analysis: The data obtained from full factorial design was subjected to analysis of variance (ANOVA) to determine significant difference among all the runs. p - value less than 0.05 was considered significant. All statistical analysis was performed using Minitab 16 (Minitab Inc, USA) statistical software package.

RESULTS AND DISCUSSION:

Isolation, identification and characterization of the Isolate: The strain was characterized for various physiological properties. The isolate was found to be Gram positive rods with slight motility. The biochemical characteristics of the strain to hydrolyze starch and ability to produce acid using carbohydrates were investigated according to Bergey's manual of Bacteriology. Catalase, Urease, Nitrate reduction tests were positive. Based on the morphological and biochemical characterization the strain was identified as *Bacillus spp*. The additional characterization by 16S rRNA sequencing revealed that it contains 812base pairs.

Blast analysis of the sequence had a high scoring similar and was identified as *Bacillus oshimensis.* The sequence of 16S rRNA was submitted to the GenBank database and can be accessed under Genebank accession number KF415293.

Growth kinetics: Growth kinetics of *Bacillus oshimensis* were studied using basal as well as optimized Horikoshi II medium. On basal medium the organism showed an extended lag phase of 8 h followed by log phase upto 24-30 h and stationary phase thereafter **(Fig. 1)**.

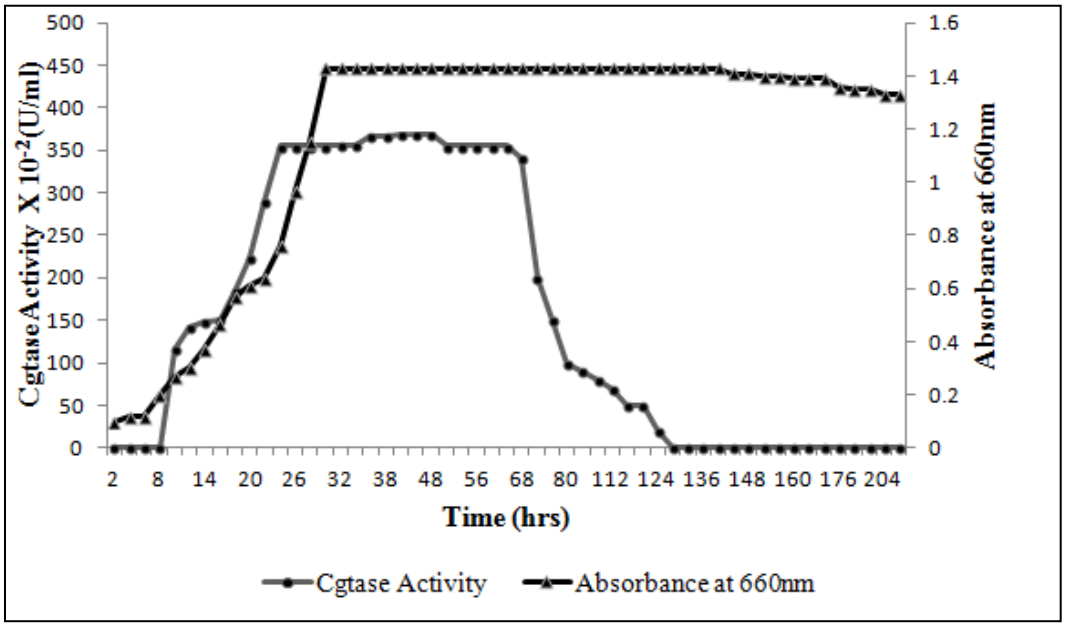

FIGURE 1: **GROWTH PROFILE AND CGTASE PRODUCTION BY *BACILLUS OSHIMENSIS* ON HORIKOSHI MEDIUM (II) CONTAININGSOLUBLESTARCH**

The CGTase production was significant during the log phase and it continued to increase. The maximum yield of CGTase was 3.25U/ml after 24 h and was found to decline after 40 h of incubation. In the optimized Horikoshi II medium the lag phase was found to be shorter than in the basal medium. CGTase production was also found to be 4.5U/ml in 24 h and remained constant till 72 h of incubation **(Fig. 2)**.

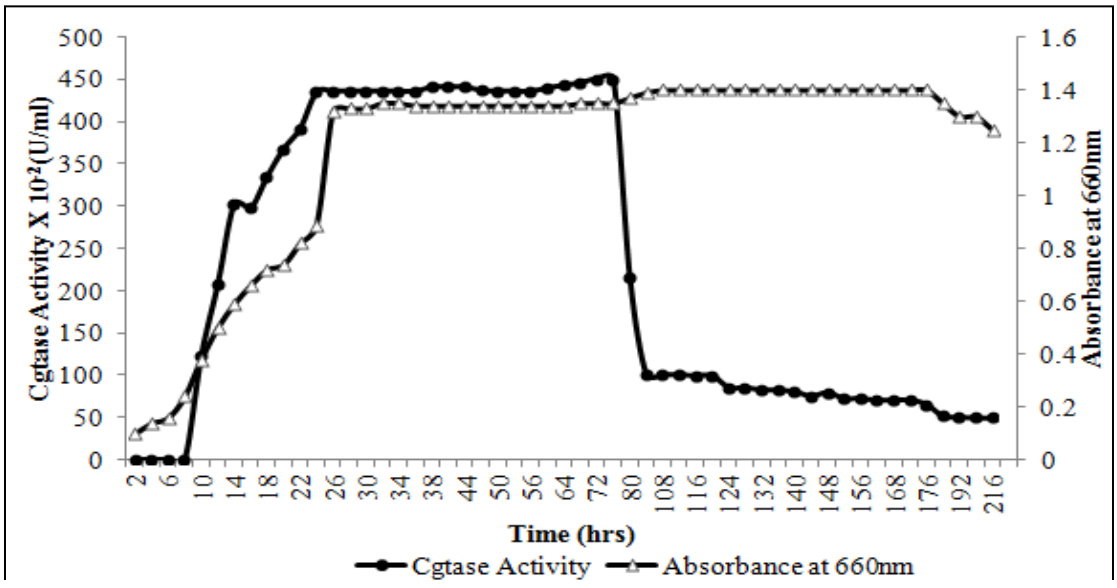

FIGURE 2: GROWTH PROFILE AND CGTASE PRODUCTION BY *BACILLUS OSHIMENSIS* ON OPTIMIZED MEDIUM CONTAINING TAPIOCA STARCH

It was found that extracellular CGTase activity in optimized medium was improved and was maintained over a period of stationary phase as compared to normal Horikoshi medium. Nogrady *et al* [20] and Posci *et al* [21] reported that the extracellular CGTase may not be involved in the degradation of starch during the exponential phase as the enzyme is released into the culture media only after all the starch has been consumed. The enzyme is anchored between the cell membrane and the cell wall and is released during the late log phase. Prolonged incubation may reduce the enzyme production probably due to the accumulation of toxic products in broth cultures that inhibit the cells from growing.

Effect of initial pH: pH influences the growth of the microorganisms as well as secretion of the enzymes in growth medium [19]. The CGTase activity was found to be optimum in the pH range from 7 – 10 with maximum activity at pH 10 **(Fig. 3)**.

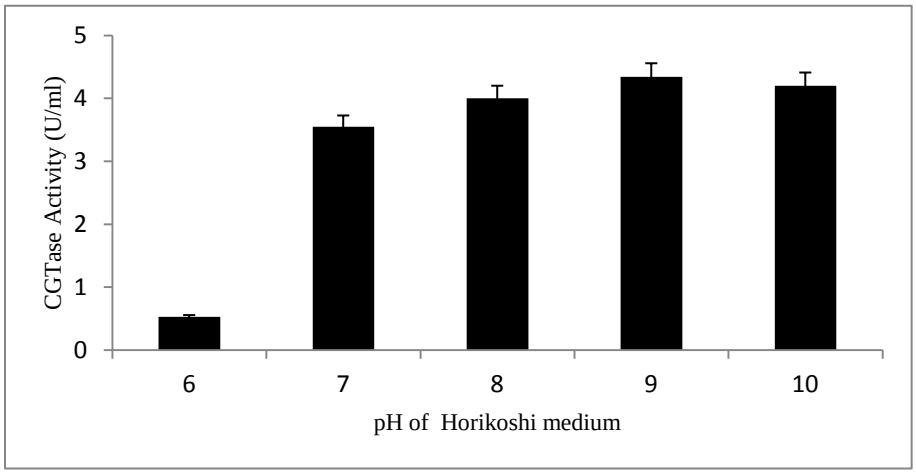

FIGURE 3: EFFECT OF INITIAL PH ON CGTASE PRODUCTION BY ISOLATED *BACILLUS OSHIMENSIS*

The maximum CGTase activity was obtained at pH 10 which corresponds to 1% Na_2CO_3. The organism was found to be alkaliphilic. Similar results were obtained by in *Bacillus licheniformis* [8], *Bacillus lehensis* S8 [22], *Amphibacillus sp.* NPST – 10 [10].

Effect of various carbon and nitrogen sources: The effect of carbon source on cell growth and CGTase production was studied using different carbohydrates. Tapioca starch was found to be the most favorable carbon source in comparison to soluble starch for CGTase production **(Fig. 4)**.

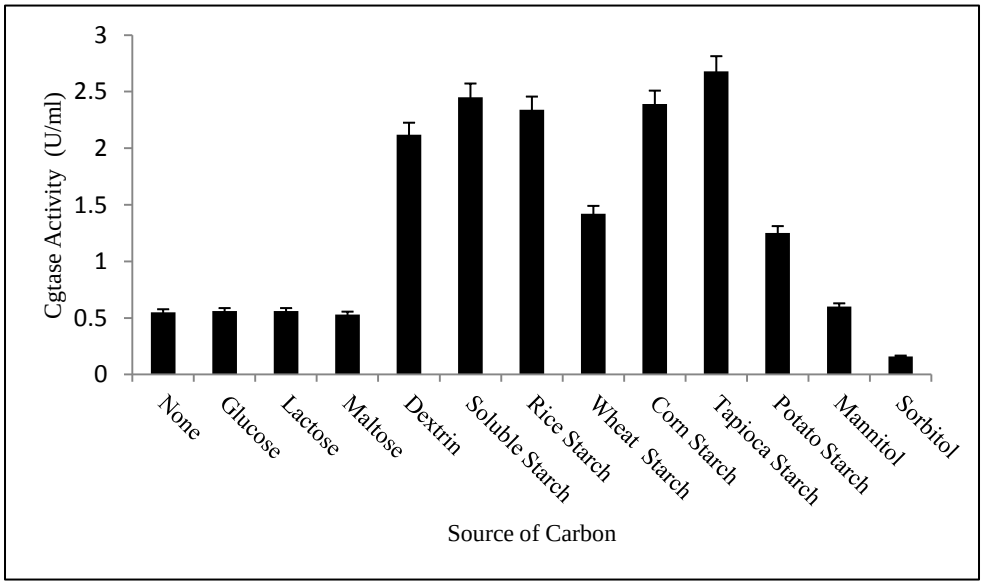

FIGURE 4: EFFECT OF DIFFERENT CARBON SOURCES AT 10G/L ON CGTASE PRODUCTION BY ISOLATED *BACILLUS OSHIMENSIS*

The concentration of carbon source is important in enzyme production by many micro-organisms especially when carbon source is required in the enzyme induction [23]. In view of this, effect of tapioca starch on CGTase production by the alkalophile was evaluated. From our findings, low concentration of tapioca starch (1% w/v) gives higher CGTase production. Illias *et al* [24] reported maximum CGTase production with 1% tapioca starch as the carbon source for *Bacillus sp.* G1. Low concentration of starch contributed higher CGTase activity than the higher concentration of starch [23].

Besides, CGTase activity was found to remain stable with 3% of tapioca starch. High concentration of carbon source reduced the CGTase production which may be due to catabolite repression occurring above a certain concentration of carbon [22].

The study of effect of organic and inorganic nitrogen sources on CGTase production indicated that the strain produced the enzyme both in presence of organic and inorganic source of nitrogen **(Fig. 5)**.

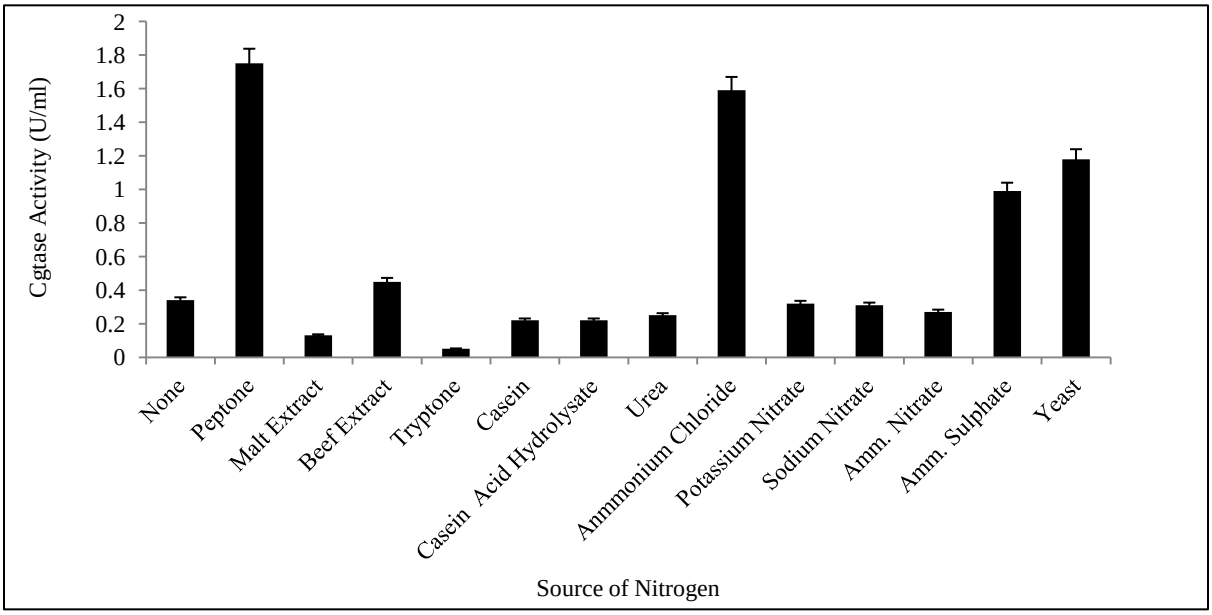

FIGURE 5: EFFECT OF DIFFERENT NITROGEN SOURCES AT 10G/L ON CGTASE PRODUCTION BY ISOLATED *BACILLUS OSHIMENSIS*

Peptone was found to be the best organic source after the yeast extract, whereas; ammonium chloride was the best inorganic source amongst the other inorganic sources used for the enzyme production [25]. The organic nitrogen compounds are good source in comparison to the inorganic sources. Soy peptone alone was able to provide the best cellular growth for the strain but the production of CGTase increased from the combined nitrogen sources. Other studies also found peptone and yeast extract to promote highest production of CGTase. The concentration of yeast extract in the medium is the most important variable for production of CGTase because it is rich in amino acids, trace elements and inorganic salts [26]. The combination with 1.5% was used to achieve high CGTase activity at shorter period of cultivation. A low level (0.75%) of nitrogen source was also reported as the optimum level for higher CGTase production than the higher level of nitrogen source. In our study, 0.5% yeast extract was set as the optimum concentration for CGTase production by the alkaliphile.

Effect of phosphorus sources and concentration of Magnesium Sulphate: Metal ions are necessary for cell growth and maintenance of active conformation of enzymes [5]. Among the different phosphorus sources tested, ammonium dihydrogen phosphate was found to be the most effective followed by disodium hydrogen phosphate **(Fig. 6)**.

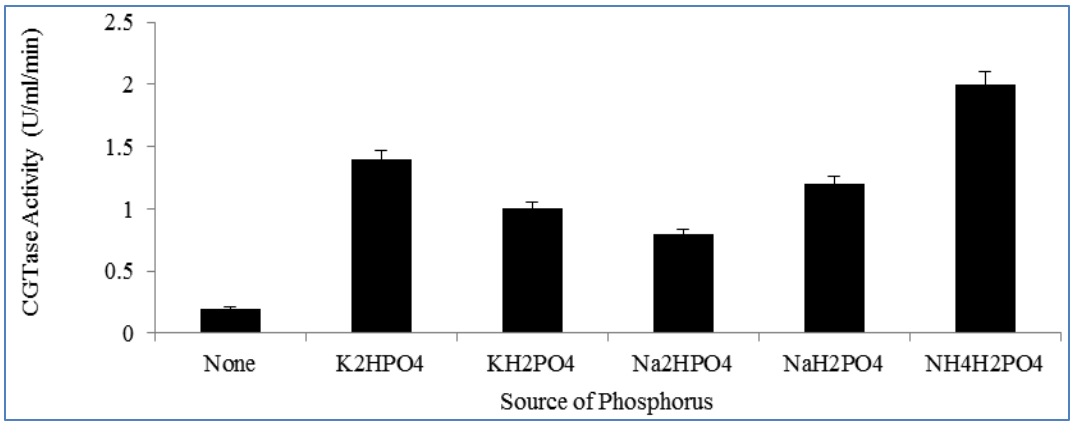

FIGURE 6: EFFECT OF DIFFERENT PHOSPHORUS SOURCES AT 1G/L ON CGTASE PRODUCTION BY ISOLATED *BACILLUS OSHIMENSIS*

Magnesium sulphate at 0.14% concentration showed highest CGTase activity **(Fig. 7)**. The literature survey reports $MgSO_4$ as the best mineral source for CGTase production. *Bacillus oshimensis* showed highest CGTase activity with 0.14% $MgSO_4$.

Experimental Design and Optimization: Fig. 8 shows the results obtained by experimental designing using Taguchi method.

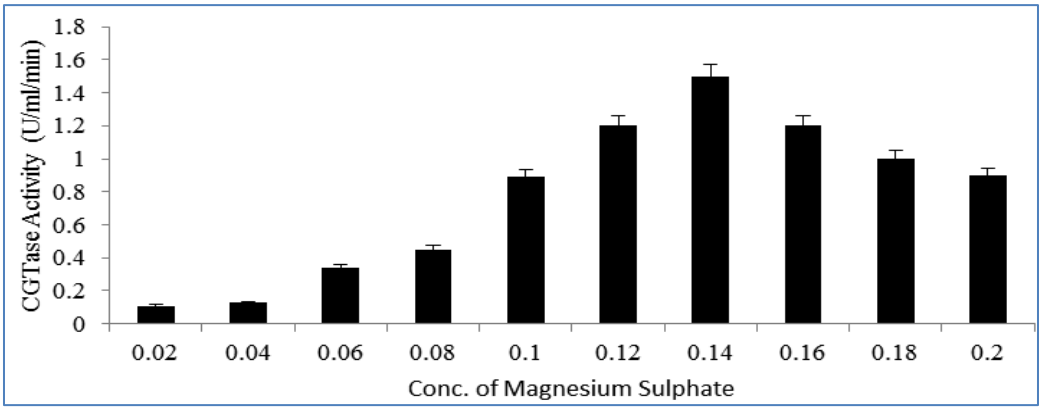

FIGURE 7: EFFECT OF DIFFERENT CONCENTRATION OF MAGNESIUM SULPHATE ON CGTASE PRODUCTION BY ISOLATED *BACILLUS OSHIMENSIS.*

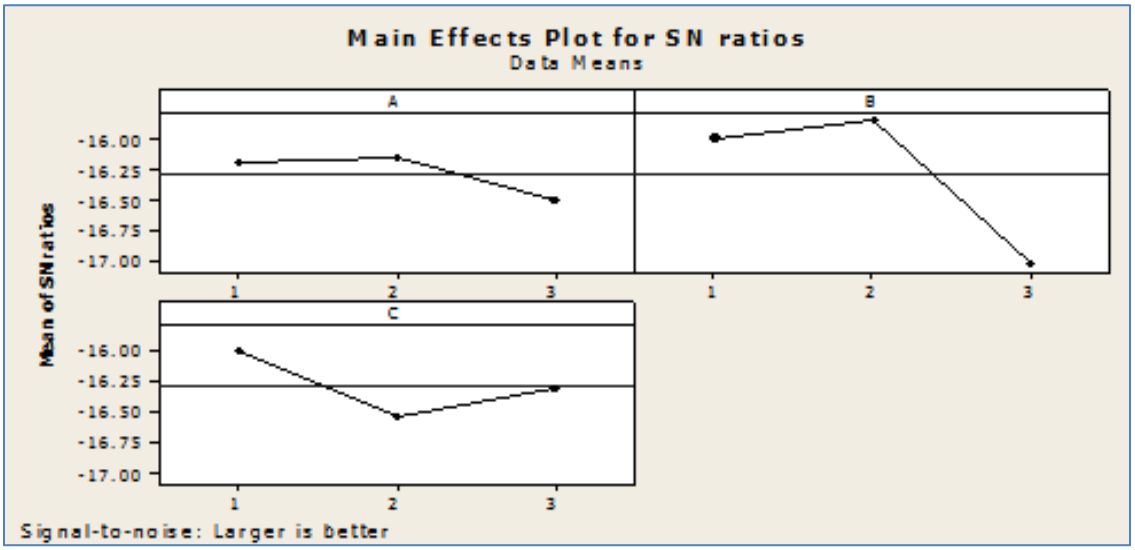

FIGURE 8: MAIN EFFECTS PLOT OF TAGUCHI METHOD FOR CGTASE PRODUCTION BY *BACILLUS OSHIMENSIS* (A: TAPIOCA STARCH, B: PEPTONE, C: YEAST EXTRACT)

It showed that peptone and yeast extract had minor effect on signal to noise ratio. Starch had virtually no effect on signal to noise ratio, which is shown by almost a flat line.

TABLE 2: EXPERIMENTAL RESULTS FOR CGTASE PRODUCTION BY *BACILLUS OSHIMENSIS* WITH TAGUCHI METHOD

Run	Tapioca Starch	Peptone	Yeast Extract	Cgtase Activity (U/ml)
1	1	1	1	3.80
2	1	2	2	3.49
3	1	3	3	3.39
4	2	1	2	3.54
5	2	2	3	3.77
6	2	3	1	3.39
7	3	1	3	3.64
8	3	2	1	3.59
9	3	3	2	3.45

The experimental results shown in **(Table 2)** were analyzed further using the analysis of variance (ANOVA). The ANOVA results are shown in **(Table 3)**.

TABLE 3: ANOVA FOR 3^3 FACTORIAL TAGUCHI DESIGN FOR CGTASE PRODUCTION BY *BACILLUS OSHIMENSIS*

Source	DF	SS	MS	F- value	p - value
Tapioca Starch	2	0.0000009	0.0000004	0.00	0.998
Peptone	2	0.0010709	0.0005354	2.78	0.175
Yeast Extract	2	0.0002142	0.0001071	0.77	0.521
Error	4	0.0007698	0.0001924		
Total	8	0.0018416			

DF: degree of freedom; SS: sum of squares; MS: mean of squares

With 95% confidence interval, the parameters have no significant effects ($p>0.05$) CGTase yield. During the present study it was observed that a cheaper carbon source i.e. tapioca starch could be used to replace soluble starch in basal medium, for obtaining a comparable amount of CGTase in the fermentation broth.

In addition, the optimized medium can be employed for increasing the production of CGTase with locally isolated *Bacillus oshimensis*.

CONCLUSIONS: The CGTase production increased using tapioca starch, which is a cheaper source of carbon in comparison to soluble starch used in the basal medium. The Taguchi method was used to design the optimization experiment in this study.

From the analysis, the optimal conditions obtained consisted of Tapioca starch 1%; peptone 0.5%; yeast extract 0.5% , 0.14% Magnesium Sulphate and 0.2% ammonium dihydrogen phosphate.

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