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Advances in Bioresearch

## ORIGINAL ARTICLE

# Production of Cellulase Utilizing Pongamia Oil Cake from *Streptomyces Xiamenensis* with Detergent Compatibility and Biopolishing property

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**ABSTRACT**
*Present work focuses on the isolation of potent cellulase producing organism/s from different soil samples and cost-effective production of the enzyme using various agricultural wastes. Enhanced production of cellulase (108.22 IU/ml) using inexpensive Pongamia Oil Cake (POC) from Streptomyces xiamenensis was obtained using POC (2% (w/v))as substrate at pH 8.0 and temperature 30°C in 48 h. Temperature 55°C and pH 6.0 showed optimum cellulase activity (122.5IU/ml). The cellulase enzyme exhibited appreciable biopolishing effect on fabrics at 45°C, pH5.0 and 19 h of the treatment in shaker conditions for 100 rpm, and showed 100% stability with Ariel® and Tide® detergents, depicting detergent compatibility with higher stability.*
**KEYWORDS:** *Cellulase, Pongamia Oil cake, Streptomyces xiamenensis, Submerged fermentation, Biofinishing, Detergent compatibility*

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## INTRODUCTION

Today our biggest concern is the safety of our environment. Industrial effluents such as dyes, chemicals and oil cakes and agricultural wastes such as leaves, bagasse, straw, husks, fruit peels, etc. are among the major pollutants as they are dumped either on farmland or water bodies making it toxic to the environment. And the best way to eradicate these pollutants is to convert these wastes into some useful products.

Oil cakes, one among the pollutants, are obtained after extraction of oil from seeds and contain several anti-nutritional and toxic components which restrict their use as fertilizers or animal feeds [1, 2]. Hence, the serious environmental threat it poses obligates its management [3]. However, some of these cakes are a rich source of nutrients, *viz.,* protein, sugars, etc. [4-8]. *Pongamia pinnata* (Karanj) is a deciduous tree, belonging to the family Fabaceae. This plant is extensively cultivated in Asian and Australian continents for their oil-bearing seeds. *Pongamia pinnata is* evaluated as a potential candidate tree for producing biodiesel in India [9]. After the extraction of oil from its seed, ~60% of the material is left as de-oiled seed cake [1]. As POC is non-edible, it cannot be used as animal feed. However, it can be used as a fertilizer [10]. POC are rich in proteins (>30%) and carbohydrates (> 30%) [11], which makes them noteworthy to be explored for their potential uses.

Among various enzymes used in industries, cellulase is considered to be one of the most important and useful enzymes [12]. It can be produced by bacteria, fungi or actinomycetes. The potential of cellulases is dominant in various industries such as textile, de-inking, pulp and paper industries, laundry detergents, winery etc. [13, 14, 15, and 16].

The present paper attempts to address the management of POC *via* submerged fermentation (SmF), producing cellulase as value added product from *Streptomyces Xiamenensis.* The cellulase thus produced was also found to exhibit promising properties such as biopolishing and detergent compatibility.

To the best of our knowledge, this is the first study undertaken to produce cellulase using POC for its application in textile and detergent industries. It is thus imperious to exploit these agro-wastes to make bioconversion processes cost effective and environmentally friendly.

## MATERIAL AND METHODS

### Sampling:

Soil sample collection was from different parts of Bangalore**.** The soil used was dark brown and sandy.

### Isolation of Microorganisms:

Serial dilution was carried out for the samples. Soil sample was taken in dilutions from $10^{-1}$ to $10^{-5}$ respectively. Dilutions were prepared to take 1g of the soil sample in 9 ml distilled water. This was used as stock and from that 100 µl of dilution were plated on nutrient agar medium incubated in room temperature at 37°C for 24 h. 25 isolates from soil were obtained.

### Screening for Cellulase producers:

The grown cultures were transferred into CMC – CR (Carboxymethyl cellulose Congo red) agar medium containing 2% (w/v) CMC to screen for the cellulase producers. Isolates giving higher Enzymatic index (≥ ) were selected for further studies.

### Phylogenetic identification:

Species identification of the selected actinomycetes sp. S5 was done by sequencing of 16s rRNA gene (Chromous Biotech Pvt. Ltd., Bangalore). NCBI-BLAST (***Basic Local Alignment Search Tool)*** and phylogenetic lineage analysis of the sequence obtained (using Phylip tool, based on neighbor joining algorithm) was also done [17].

### Enzyme production and assay:

The selected isolates were grown in nutrient agar plate to build the inoculum for the assay. Two loopful from each built-up inoculum was transferred to minimal media with 1% (w/v) CMC, incubated for 3 consecutive days. The enzyme extract (supernatant) obtained by centrifuging the cultured broth at 10,000 rpm for 15 min at 4°C was used as an enzyme source for the assay. Dinitrosalisylic acid (DNS) method was used to measure the reducing sugars using glucose as standard [18]. One Unit of the enzyme is defined as the amount of enzyme required to liberate 1µM of glucose in 1 min under the said assay conditions. The isolate showing maximum cellulase titer was selected for optimization studies.

### Evaluation of different Agro-wastes for cellulase production:

To reduce the cost of cellulase production, different agro-wastes were tested as carbon source in the fermentation medium. Six different agro-wastes (1% w/v) – Groundnut Oil cake (GOC), Rice husk (RH), Jowar Bran (JB), Pongamia Oil cake (POC), Corn Cob (CC), Jatropha Oil Cake (JOC), were used for cellulase production replacing CMC in the production medium The agro-waste supporting highest cellulase production was taken for further optimization studies using "One factor at a time" approach.

### Factors affecting Cellulase production:

Five different physical and nutritional factors – different concentrations of POC (0.2-2.0 % w/v ) different Nitrogen sources (Peptone, Corn Steep Liquor, Soya bean Meal, $NH_4Cl_2$, $NaNO_3$, Urea and $(NH_4)_2NO_3$ at 0.03% w/v), pH (4.0-10.0), different temperatures (30-60°C) in fermentation medium and different incubation time (24-120 h) were evaluated for the study.

### Determination of pH and temperature optima of cellulase:

The optimal pH of cellulase was determined by assaying activity using the solution of 1 % CMC in 0.1 M buffers of different pH values (4.0–10.0) as a substrate. pH stability of the enzyme was checked at the optimum pH for different time intervals (0-120 min) at optimum temperature. The absorbance was read at 540 nm.

Similarly, to determine the temperature optima for cellulase the activity was tested in different temperatures 35°C-65°C and room temperature (28°C). The temperature stability of cellulase was determined by incubating the reaction mixture for different time intervals (0, 60, 90 and 120 min) at the optimum temperature followed by the assay at optimum pH and temperature by DNS method [18].

### Bio-finishing (Weight loss):

Desized fabrics were subject to biotreatment using cellulase enzymes. The biotreatment was conducted with a material-liquor ratio of 1:50. Five steel balls were added to each gram of the fabric tested. The biofinishing experiment was carried out at different concentrations of cellulase (1 - 5 % (w/v)) and temperatures (40 - 80ºC) for 19 h. Later, the temperature of the system was increased to 100ºC for 10 minutes to stop the enzyme action. The fabrics were then washed with hot water and then cold water and dried in ambient conditions [19].

**Detergent stability:**

Different detergents (Ariel®, Surf excel®, Tide®, and Patanjali detergent) were diluted to a final concentration of 7mg/mL (0.7g/10 mL). The detergents were taken in test tubes. The inherent enzymes of the detergent were inactivated by heating the tubes with detergents to 100°C for 15 minutes. A combination of buffer and the crude enzyme was taken as a control to compare the test. After inactivation, the tubes were allowed to come down to room temperature. From this reaction mixture, 2 mL was taken for the cellulase assay.

## RESULTS AND DISCUSSIONS

**Isolation and screening of cellulase producers:**

Leaf litter and soil samples which are rich sources of cellulase producing microorganisms were obtained from in and around Bangalore. The cellulase producing organisms were isolated from different samples by serial dilution method and spread plating on CMC agar. The screening of the cellulolytic bacterial isolate was performed based on the diameter of the clearing zone surrounding the colony on the CMC medium (Table 1).

Out of the 25 isolates obtained, 7 were positive for cellulase production and negative for amylase production and were carried forward for enzyme assay. Among these, isolate S5 showed the highest enzymatic index of 2.5 (Figure 1).

**Table 1 Screening for cellulase positive isolates**

| S. No | Sample | Isolate | Zone of clearance | Enzymatic Index (cm) |
|---|---|---|---|---|
| 1 | Leaf litter | L1 | + | 1.3 |
| | | L2 | + | 0.9 |
| | | L3 | - | |
| | | L4 | - | |
| | | L5 | - | |
| | | L6 | - | |
| | | L7 | + | 0.8 |
| | | L8 | - | |
| | | L9 | + | - |
| 2 | Soil | S1 | - | |
| | | S2 | - | |
| | | S3 | - | |
| | | S4 | - | |
| | | S5 | + | 2.5 |
| 3 | Pot Soil | P1 | - | |
| | | P2 | - | |
| | | P3 | - | |
| | | P4 | + | 1.4 |
| | | P5 | - | |
| | | P6 | - | |
| | | P7 | - | |
| | | P8 | + | 1.0 |
| 4 | Rotting wood | R1 | + | 1.9 |
| | | R2 | - | |

 

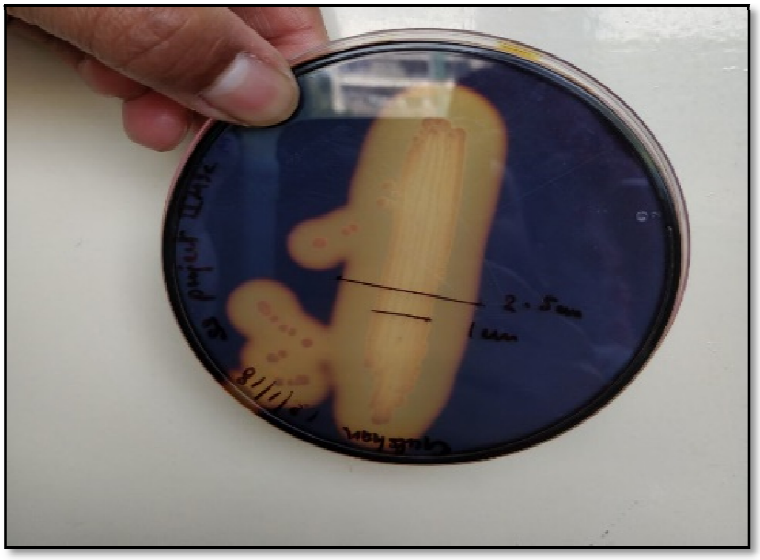

**Figure 1: Isolate S5 showing cellulase activity on CMC agar**

**Phylogenetic identification of S5:**

Based on its cultural and morphological properties (colorless with white aerial mycelium forming ovoidal spores on lateral branches of aerial hyphae and musty or earthy odor), the isolate $A_8$ was identified as *Streptomyces* sp. Further, the BLAST and dendrogram analysis of the partial 16S rRNA gene sequence (620 bp) revealed *Streptomyces xiamenensis* strain MCCC 1A01550 to be the closest relative of actinomycetes sp. $A_8$. The partial 16S rRNA gene sequence of strain $A_8$ was submitted to the NCBI database (Accession No JX827497) (Figure 2).

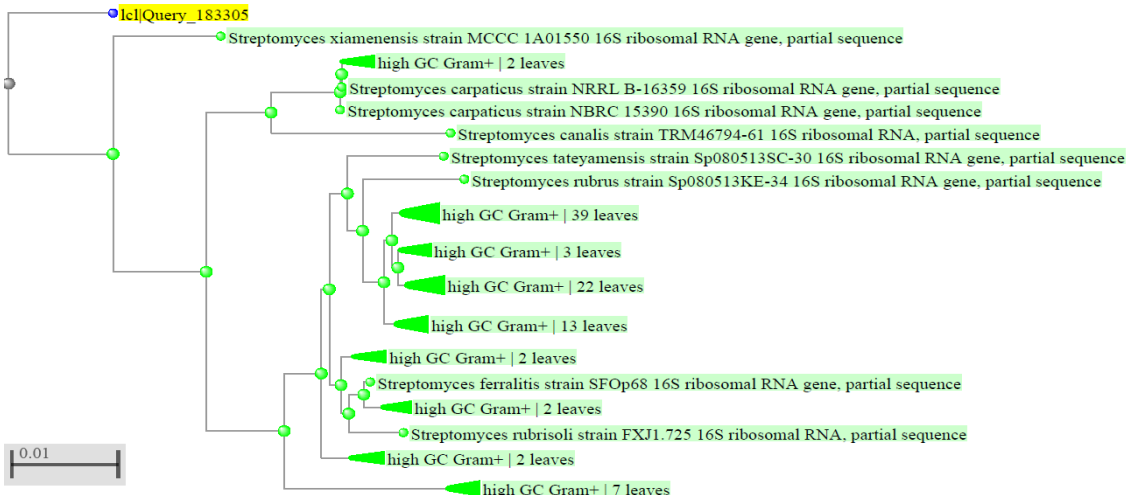


**Figure 2: Phylogenetic tree for *Streptomyces* species based on 16srRNA sequencing.**

**Enzyme production and assay:**

The culture filtrates obtained from the isolates $L_1$, $L_2$, $L_7$, $L_9$, $S_5$, $P_4$, $P_8$, $R_1$ showed cellulolytic activity (CMCase activity). Upon estimating the reducing sugar content from the selected isolate [20], it was found that the isolate $S_5$ gave cellulase activity of 10.75 IU/mL.

**Time course cellulase production and assay by isolate S5**

Cellulase production by the selected isolate $A_8$ was quantitatively determined in a time course manner at an interval of 24 h by growing the isolates on NB containing CMC 1% (w/v) and determining cellulase activity **(Figure 3).** $A_8$ on 1 % (w/v) CMC showed a range of cellulase activity in a time-dependent manner with the highest activity of 12.06 IU/ml on Day 3 which was used for further studies in the enzyme production and explored for its bio-finishing activity. Incubation beyond the optimum time demonstrated a fast decrease in the enzyme activity, as compared to maximum. It may be because of the exhaustion of supplements in the fermentation medium leading to inactivating enzyme machinery [21].

Similarly, 10 days incubation period was essential for cellulase production in *Bacillus* sp. [22], whereas Fawzya et al.,2013, [23] worked with *S.marcescens* SGS 1609 isolate which showed maximum cellulase production by 4th day .

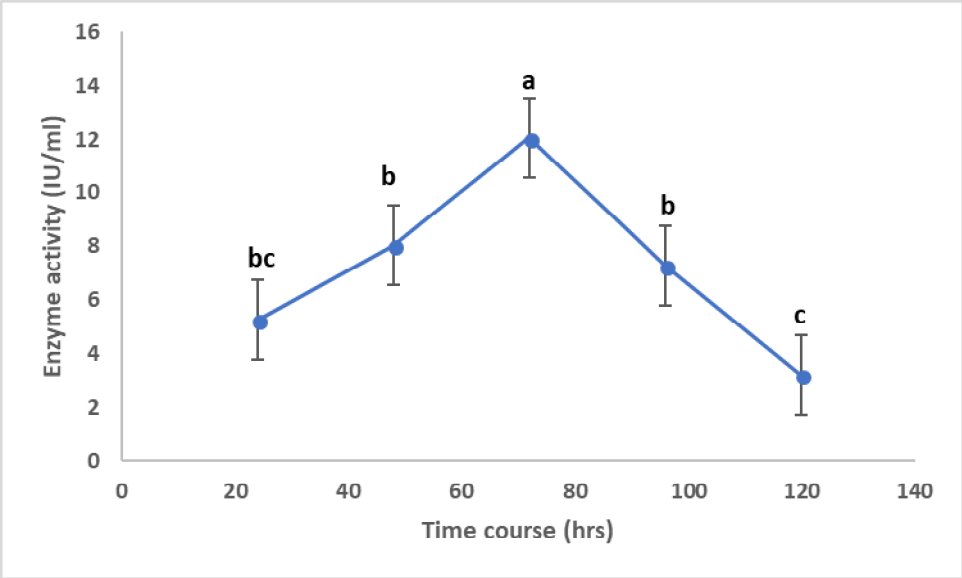


**Figure 3: Time course Cellulase activity of S5 on basal media**

**Evaluation of different agro-wastes for cellulase production:**

Six different agro-wastes were explored for cellulase production by $A_8$. All substrates supported varied levels of cellulase production by 72 h. Among the different substrates tested, Pongamia Oil Cake (POC) supported the highest cellulase activity of 45.99 IU/ml. (Figure 4).

One of the important criteria taken into account for the choice of pongamia oil cake for its cost effectiveness, easy availability and richness in cellulose [24].

At 10 % concentration of molasses, *Bacillus subtilis* exhibited maximum activity [25].

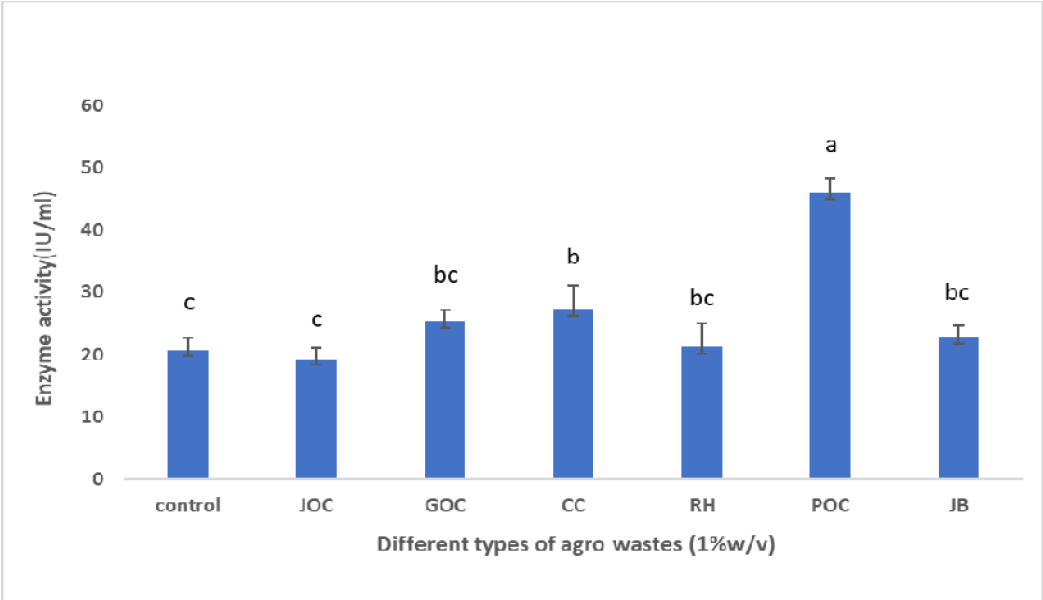


**Figure 4: Effect of different agro-wastes (1%) on cellulase production**

**Factors affecting Cellulase Production using POC:**

**Time course:**

The CMCase activity of *S. xiamenensis* taken on each day of the incubation period (24-120 h) revealed that highest enzyme activity (83.33 IU/mL of CMCase activity) was shown on Day 2 (Figure 5). From Day 3 onwards, there was a rapid decline in the same, which could be due to depletion of nutrients and accumulation of toxic products [26].

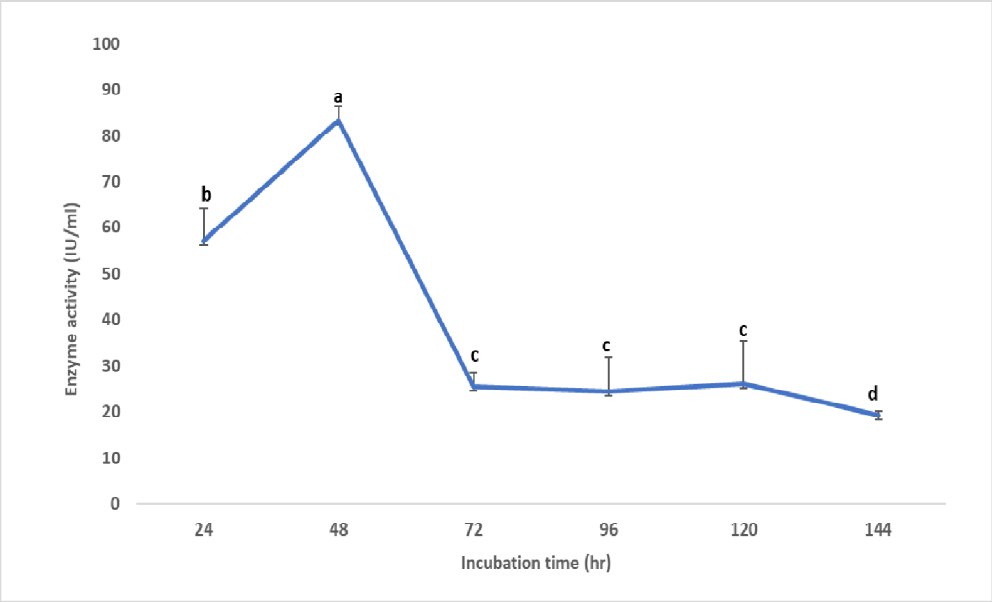


**Figure 5: Effect of incubation period on the production of cellulase by *S. xiamenensis*.**

**Effect of pH on cellulase production**

The optimum pH for maximum enzyme production was 8, with 89.64IU/ml of CMCase activity.

The enzyme activity gradually increased when increasing the pH with optimum pH of 8.0 followed by a gradual fall in activity (Figure 6).

Song *et al*., [27] observed optimal cellulase production at pH 9.0 by *Clostridium acetobutylium.*

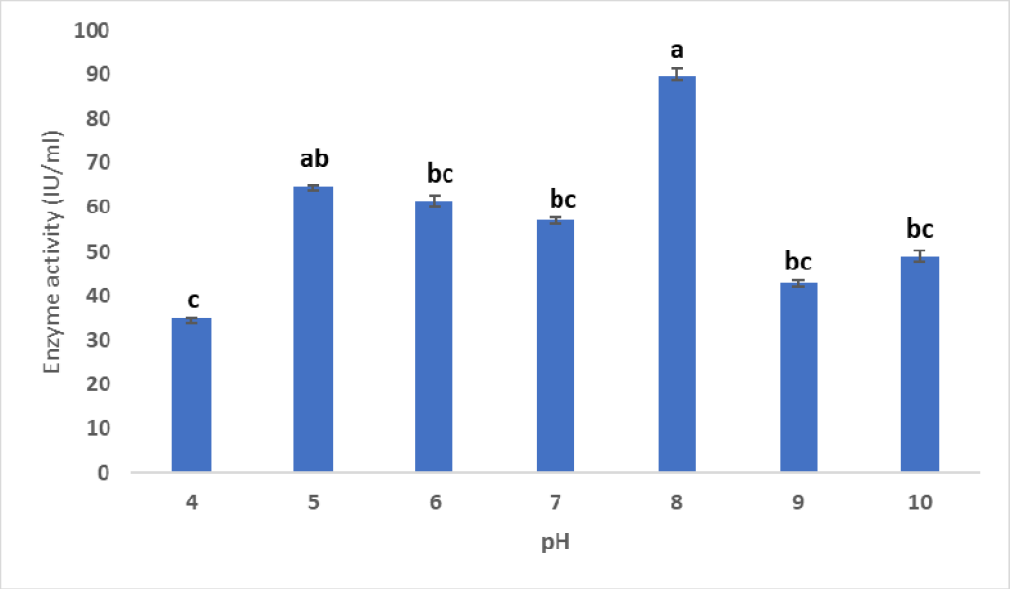


**Figure 6: Effect of pH on cellulase production by *S. xiamenensis***

**Effect of temperature on production of cellulase:**

The cellulase production by *S. xiamenensis* was observed to be the highest at 30°C (87.8IU/ml of CMCase activity) (Figure 7). There was a sharp decrease in enzyme production with a further increase in temperature. Similarly, *Streptomyces drozdowiczii* was cultivated at 30°C and resulted in the highest CMCase level of 595 U/l [28].

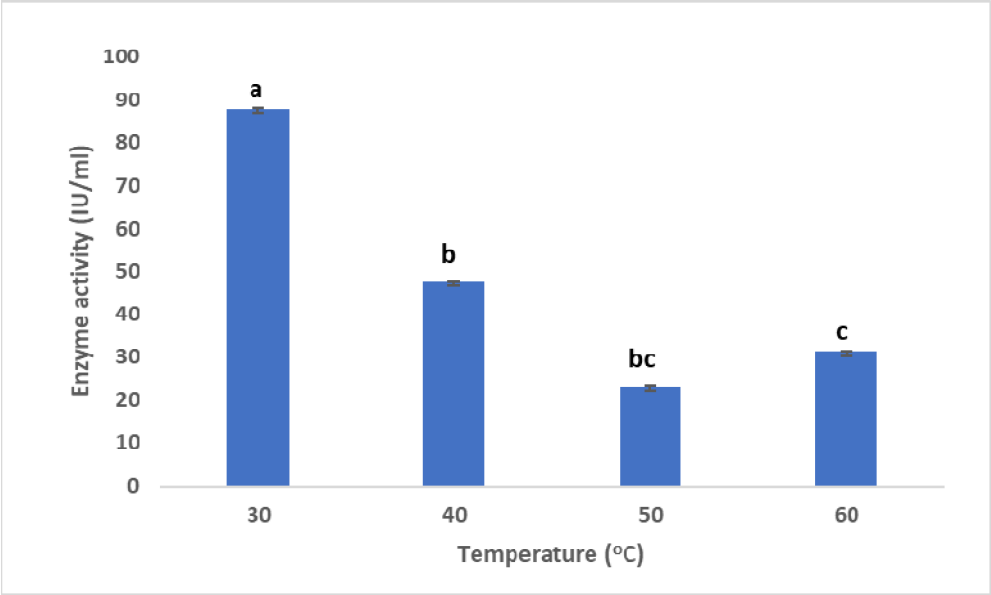


**Figure 7: Effect of incubation temperature on production of cellulase by *S. xiamenensis***

**Effect of POC concentration on cellulase production:**

When *S. xiamenensis* was grown using different concentrations of POC (0.5-3.0%), the production of cellulase increased with increase in POC concentration up to 2% (108.22IU/ml). Beyond this point, there was no significant change in production and an almost linear plateau was achieved (Figure 8).

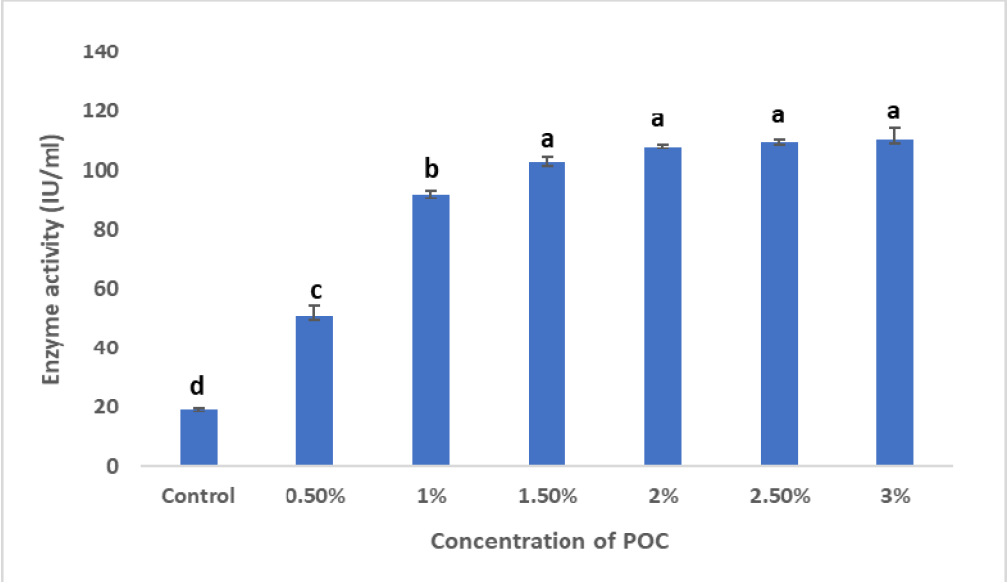


**Figure 8: Effect of substrate concentration (POC) on CMCase activity by *S. xiamenensis***

**Effect of different Nitrogen sources on cellulase production:**

To determine the best Nitrogen source, the culture filtrates were assayed for reducing sugar content. It was found that the nitrogen source in the basal media, $(NH_4)_2NO_3$ continued to remain the best nitrogen source for enzyme production (Figure 9). Compared with various organic nitrogen and inorganic nitrogen sources, $(NH_4)_2NO_3$ supported least biomass and higher cellulase activity, attributing to the fact that complex substances in organic nitrogen sources could trigger the biomass production, making it unnecessary for the organism to produce cellulase [29].

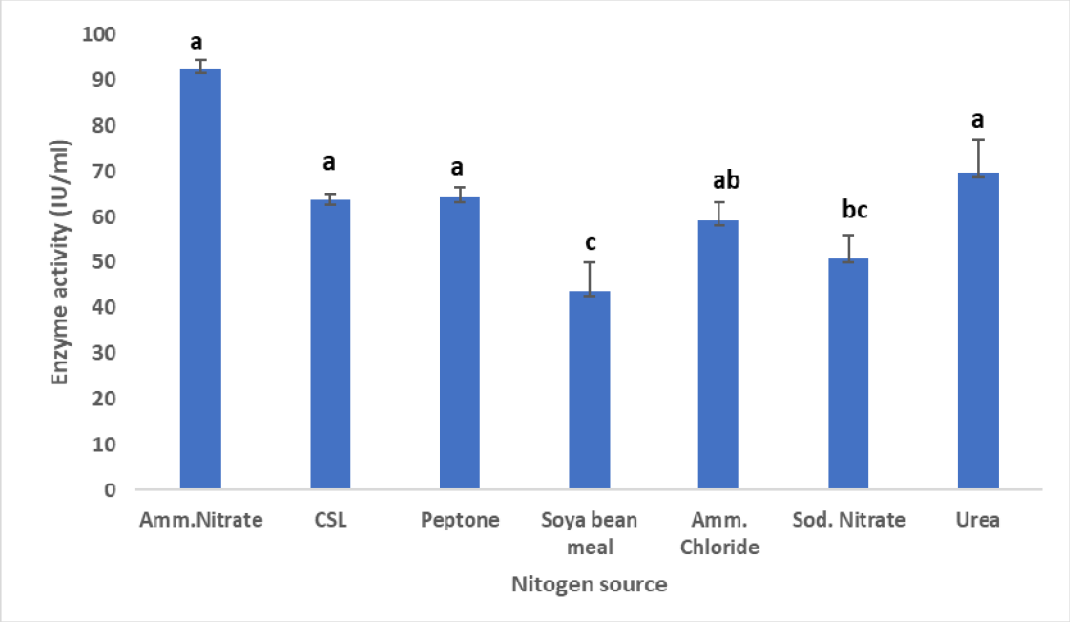


**Figure 9: Effect of different nitrogen sources on the production of cellulase by *S. xiamenensis***

**pH and temperature optima of Cellulase activity:**

**Effect of pH on the activity of cellulase enzyme:**

The optimum pH for the activity of the cellulase isolated from *S. xiamenensis* was found to be pH 6, with an enzyme activity of 126.3IU/ml, after which there was a gradual decline in enzyme activity(Figure 10).

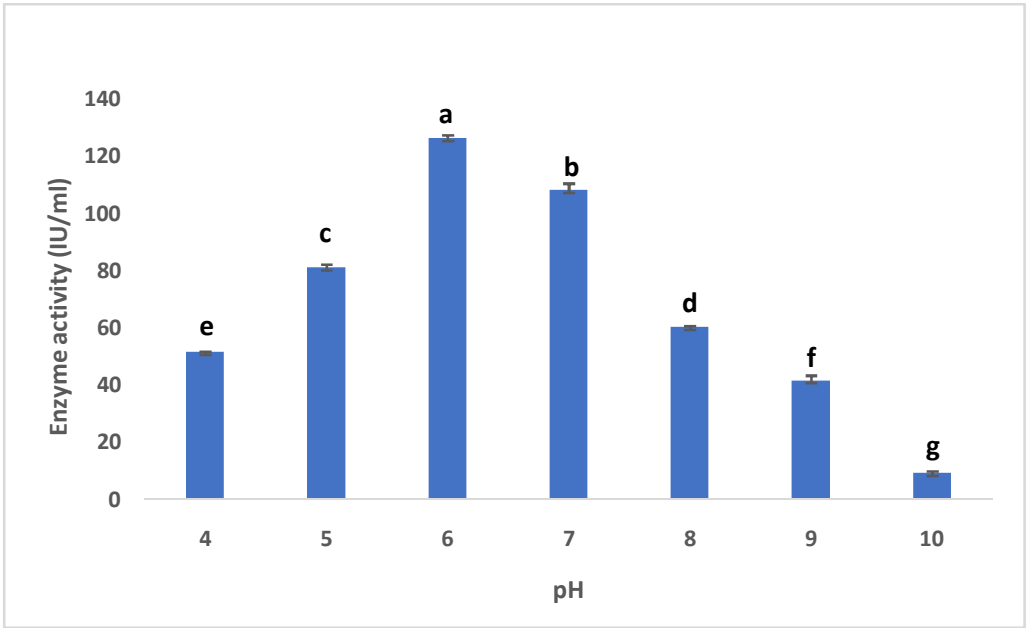


**Figure 10: pH optima of *S. xiamenensis* cellulase**

**Effect of Temperature on the activity of cellulase enzyme:**

The cellulase from *S. xiamenensis* showed maximum enzyme activity (CMCase activity) of 122.5IU/ml at a temperature of 55°C. This was the optimum temperature after which there was a steep decline in activity (Figure 11). This is comparable to that of cellulase from *Streptomyces drozdowiczii*, which showed maximum CMCase activity at 50°C [30].

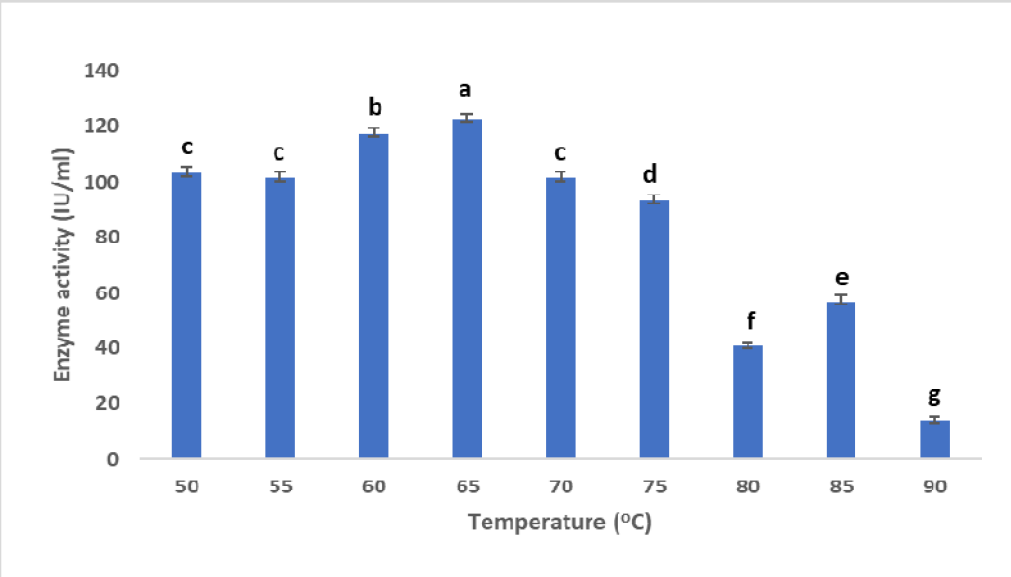


**Figure 11: Temperature optima of *S. xiamenensis* cellulase.**

**Bio-finishing of cotton:**

Post enzymatic treatment of the fabric, it was observed that enzyme-treated fabric had a softer feel upon touch and appeared to have fewer pills and fibrils. Both the cellulase treatments- CMC grew and POC grown showed an appreciable weight loss of the fabric indicating the bio-finishing potential of the enzyme at 45°C, pH 5.0 and 19 h of the treatment in shaker conditions for 100 rpm**(Table 2).**

**Table 2: Weight loss of fabric on cellulase treatment**

| Treatments | Weight loss (g) | Percent weight loss (%) |
|---|---|---|
| Control (w.o. enzyme treatment) | 0.02 | 3.33 |
| CMC based cellulase | 0.15 | 19 |
| POC based cellulase | 0.24 | 30 |

**Detergent stability:**

The compatibility of cellulase with some commonly used detergents was tested with a view to exploiting the enzyme in the detergent industry. Addition of cellulases in the detergent compositions is a new trend followed by many detergent industries in recent years. Alkaline cellulases present in the detergent composition can pass through the inter-fibril spaces easily, effectively removing stains from textiles. Additionally, cellulases process cellulose fibrils, and impart color brightness and smoothness to clothes, even after repeated washing [30].

The compatibility of the enzyme varied for each laundry detergent, with higher stability being observed in the presence of Surf Excel® and Patanjali detergent. The enzyme also showed 100% stability with Ariel® and Tide® (Table 3). These findings suggest that the performance of an enzyme in detergents depends on a number of factors, including the compounds used in commercial formulations.

**Table 3: Detergent stability of cellulase of *S. xiamenensis* grown on POC**

| Detergent/ Sample | Surf Excel® | Ariel® | Tide® | Patanjali detergent |
|---|---|---|---|---|
| Active Detergent without Enzyme | 32.08 IU/mL | 8.75 IU/mL | 11.66 IU/mL | 17.5 IU/mL |
| Enzyme from POC + Inactivated Detergent | *29.16 IU/mL | *23.33 IU/mL | *23.33 IU/mL | *29.16 IU/mL |
| *Relative activity with respect to Enzyme Activity of cellulase from POC being 23.33 IU/mL (taken as 100%). | | | | |

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