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Production and Optimization of Xylanase by Thermophilic *Bacillus* sp. Isolated from Soil

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Totally 15 strains of thermophilic *Bacillus spp.* were isolated from soil and five of the thermophillic bacteria showed xylanolytic activity on secondary screening based on the diameter of the clear zone formation on congo red xylan agar. On the basis of morphological, cultural, biochemical and by molecular characteristics the potential xylanolytic *Bacillus* sp. was identified as *Bacillus pumilus*. For maximum xylanase production *B. pumilus* required an optimum temperature of 50^{0}C for 72 h. The optimum pH observed was 7.0 ± 0.2. Xylan concentration at 1.0% in the production medium was found to be optimum. Production media with 1% galactose as carbon source, 1% beef extract as nitrogen source and 0.5% $FeCl_3$ as mineral salt enhances the xylanase production.

Key words: Xylanase, Thermophillic, *Bacillus pumilus.*

Microbial enzymes such as xylanases enable new technologies for industrial processes. Xylanases hydrolyze complex polysaccharides like xylan. Research during the past few decades has been dedicated to enhanced production, purification and characterization of microbial xylanase. Xylan is the most abundant renewable non-cellulosic polysaccharides present on earth. It is a major constituent of plant cell wall and constitutes around 20-30% of dry weight of tropical hard wood and annual plants. Studies reveal that xylan forms an interphase between lignin and other polysaccharides (Dhiman *et al*, 2008). It is mainly present in the secondary cell wall and is covalently linked with lignin phenolic residues and other polysaccharides such as pectins and glucans.

The microorganism that produces xylanases and other glycosides has been found in extremely diverse natural habitats. Under mesophilic growth conditions xylanolytic activity has been reported in a wide variety of different genera and species of bacteria, yeast and fungi (Gikes *et al*., 1991). Although many fungi are xylanase producers, their large scale cultivation is often difficult because of slow generation time, coproduction of highly viscous polymers, and poor oxygen transfer. Bacillaceae are used more extensively than other bacteria in industrial fermentation, since they excrete most of their enzymes (Bernier *et al*., 1983). Use of xylanase from microorganism at temperature above 50°C and in alkaline conditions is especially desirable for kraft and pulp treatment in the paper industry (Farel and Skerker., 1992).

Phenotypically, the genus *Bacillus* is a large and heterogeneous collection of aerobic, rod-shaped, gram positive (to gram-variable), endospore-forming bacteria. The diversity that exists in this genus is demonstrated by the enormous range of genomic guanine-plus-cytosine contents (32 to 69 mol %), as well as the variety of interesting phenotypes, that are found in the various *Bacillus* species. These phenotypes include (but are not limited to) the

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ability to fix molecular nitrogen and growth under extreme conditions, including growth in thermophilic, psychrophilic, acidophilic, alkalophilic, and halophilic environments, (Wisotzkey *et al., 1992*). Many *Bacillus* species also utilize a wide assortment of carbon sources for heterotrophic growth, ranging from methanol to complex natural polymers (Holt *et al*., 1984). Even facultatively autotrophic hydrogen-oxidizing sporeformers have been isolated and studied. After 1990, 16S rRNA (rDNA) has been successfully applied in determining phylogenetic relationships among *Bacillus* species (Wang and Sun, 2009).

The thermophillic microorganisms are industrially important because of their high growth rates and minimal chance of contamination. Higher thermotolerance of the enzymes is desirable for their commercial applications. However, the main problem faced by the pulp and paper industry while using enzyme treatment is the availability and the cost of the enzyme. About 30-40% of the production cost of many industrial enzymes is accounted by the cost of growth of substrate. The use of low cost substrate for the production of many industrial enzymes is one of the ways to greatly reduce the production cost. Extracellular enzymes are of particular interest because they are generally more stable and are easier to purify than intracellular enzymes, thereby reducing the cost of production. For commercial application, xylanases should ideally be produced quickly and in large quantities by the optimization of various production parameters such as temperature, time, pH, carbon and nitrogen sources. These parameters seem to enhance product recovery as well as high enzyme productivity.

MATERIALS AND METHODS

Sample collection and processing

The soil samples (1.0gm) collected from different sites of Sam Higginbottom Institute of Agriculture, Technology & Sciences -Deemed-to be- University, Allahabad, India, were dissolved in 9 ml of sterilized ringer solution and this suspension was serially diluted upto10^{-5} dilution. Further 0.1 ml of the diluted sample was spread plated over the nutrient agar media and incubated for 48 hours at 50 ± 1^0C (Corderio *et al*., 2002). Screening of xylanase producing thermophillic bacteria.

The isolated bacteria were further spot inoculated on congo red xylan agar for detection of xylanolytic activity which was indicated by formation of zone around colonies.

Identification of bacterial strain

The bacterial strains showing xylanolytic activity were identified as per cultural, morphological, biochemical (Holt *et al*., 1989) and molecular characteristics (Bosshard *et al*., 2003).

Molecular Analysis for xylanase producing *Bacillus pumilus*

DNA extraction, amplification, physical analysis and sequencing of the amplified products were done in Chromous Biotech Private Limited, Bangalore, India. 16S rRNA sequences received were further identified using Bioinformatics tools like BLAST (Basic Local Alignment Search tool) to confirm its similarity at molecular level (Bosshard *et al*., 2003).

Xylanase production

For xylanase production standard media was prepared according to the composition given by Corderio *et al*., (2002). The standard production media was composed of (g/L) xylan 10.0, yeast extract 2.0, peptone, 5.0, $MgSO_4$ 0.5, NaCl 0.5 $CaCl_2$ 0.15. The pH was adjusted to 7.0 and the medium was sterilized by autoclaving at 15 psi for 20 minutes. This medium (50 ml in 150 ml Erlenmeyer flasks) was inoculated with 2 ml of overnight culture and incubated at 50^0C with vigorous aeration in a shaker at 150 rpm. Before assay, the cells were separated by centrifugation at 10,000 rpm at 4^0C for 15 minutes. The clear cell free supernatant obtained was stored at 4^0C till further use.

Xylanase assay

The crude enzyme obtained after centrifugation was assayed for xylanase activity according to the DNS method described by Miller (1959). Xylan (1%) substrate solution was prepared in phosphate buffer of pH 7. The crude enzyme (0.2 ml) and substrate solution (1.8 ml) was added and incubated at 50^0C for 30 min in water bath. After incubation 2 ml DNS reagent was added and the reaction was stopped by boiling the reaction mixture at 100^0C for 10 min. The

reducing sugar liberated was estimated spectrophotometrically at 540 nm using D xylose as a standard. One unit of xylanase activity was defined as the amount of enzyme that produces 1 ¼mole of xylose equivalent per minute under the assay conditions.

Optimization of Production parameters

The optimization of the growth conditions were carried out based on stepwise modification of the governing parameters for xylanase production. The effect of cultivation temperature on the enzyme production was examined at different temperatures starting from 30°C to 70°C with 10°C intervals. The effects of incubation period were evaluated by 24 h interval by checking the enzyme activity. Changing the pH 5 to 10 in the production medium the effect of pH was observed. Effect of substrate concentrations (0.5% to 2.5% xylan) was examined. The effect of different mineral salts (0.5%w/v) such as KCl, $ZnSO_4$, $FeCl_3$, $MgCl_2$ and $CuSO_4$ was observed. The effect of supplementation of additional carbon and nitrogen sources to production medium was examined using carbon sources (1%w/v) like arabinose, maltose, sucrose, galactose and glucose. Nitrogen sources (1% w/v) like organic (beef extract, tryptone) and inorganic ($NaNO_3$, KNO_3, NH_4NO_3) were also tried.

RESULTS AND DISCUSSION

Isolation and Identification of *Bacillus pumilus*

Out of 15 thermophillic bacterial isolates, 5 were selected for xylanase production on the basis of clear zone formation on congo red xylan agar. Bacterial strain showing xylanolytic activities were identified as *B. pumilus* and further confirmed by computational analysis. Gene sequences of 16S rRNA of strain was compared with nucleotide database of NCBI webserver through BLAST tool. Result showed that query sequences were best pairwise aligned with 16S rRNA sequences with sequence similarity and identity of 99%. The query sequences of isolated strain (Strain No.YK02_27F) was identified as *Bacillus pumilus* strain NMSN-1d (Fig.1) and submitted in NCBI Gene data bank (accession No. FN667871).

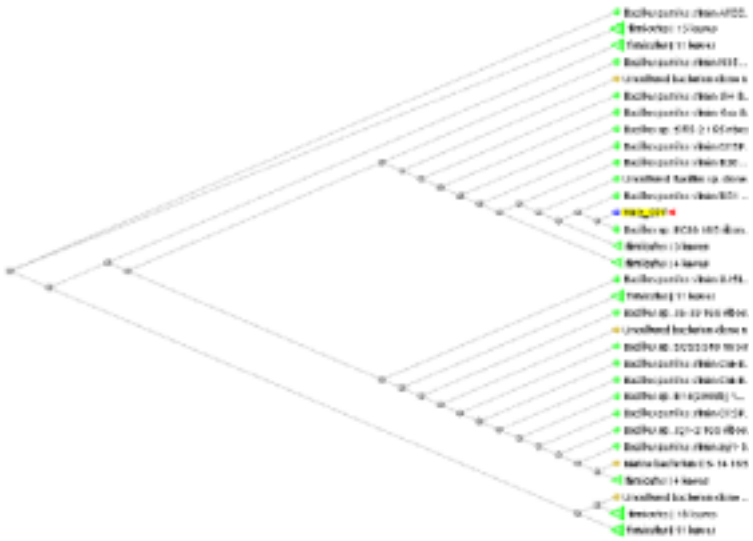

Fig. 1. Xylanase from the strains of *Cellulomonas uda, Microbacterium ulmi, M. xylanilyticum, Bacillus firmus, B. thermoleovorans, B. flavothermus, Bacillus pumilus, Bacillus sp.,* and *Streptomyces sp*. has been reported in previously conducted studies (Rapp and Wagner, 1996; Duarte *et al*., 2000; Beg *et al*., 2001; Archana and Satyanarayana, 2003; Chang *et al*., 2004; Rawashdeh *et al*., 2005). The different thermophillic *Bacillus* sp. capable of producing xylanase were also reported by Corderiro *et al*., (2002). The isolates were reported to be of *B. denitrificans, B. flavothermus B. stearothermophillus* and *B. thermolevovorous*. The production of thermophillic xylanase by *Bacillus* sp. is comparable with the present study. However, at the species level a difference was observed. This could be mainly due to the variation in source of isolation

Effect of temperature and time on xylanase production

Effect of temperature varying from 30°C to 70°C were evaluated for the detection of optimum temperature required for the production of enzyme, and the result showed optimum at 50°C (7.852 U/ml) and minimum was at 70° C (0.873 U/ml) (Fig 2). At 60°C, a significant decline in xylanase activity was found. The time course of xylanase production was investigated and maximum production was observed after 3 days (72 hrs) (7.852 U/ml at 50°C, 7.801 U/ml at 40°C) while minimum production was noted at 24 hrs (7.320 U/ml at 40°C, 7.131 U/ml at 50°C) (Fig 1). Further incubation after this did not show any increase in the level of enzyme production. Thus optimum temperature and time combination for the xylanase activity was thus found to be at 50°C and 72 h. respectively. The temperature and incubation time relation with xylanase activity were found to be statistically significant ($P < 0.05$).

Optimum temperature (50^0C) for xylanase production determined in the present study is comparable with the studies of Rawashdesh, (2005). Similarly the wide range of temperature in which xylanase was found to be active was observed to be in the range of 30-60^0C in the present study. This is in agreement with the studies of Anuradha *et al.* (1999), Loyn *et al.*, (2000) and Corderiro *et al.*, (2002). However, the optimum temperature for xylanase production by *Bacillus* strain 41M observed in the studies of Nakamura *et al.*, (1993) as 55^0C which is slightly higher in comparison with the present study. The variation in the optimum temperature for xylanase production may be due the difference in the strain used for the study.

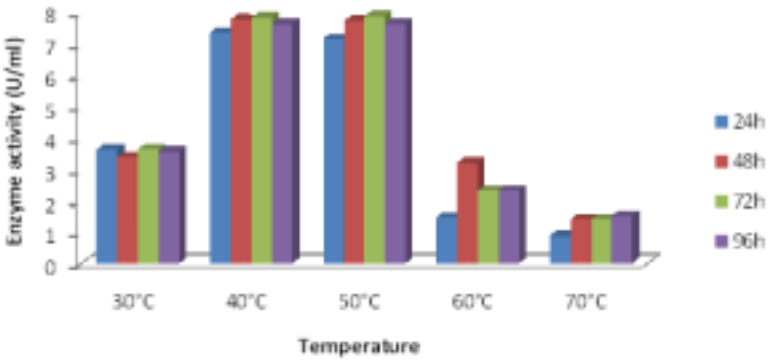

Fig. 2. Pattern of enzyme activity with respect to temperature and time

Since temperature affects the growth of a particular strains as well as the metabolic rate of organism, the variation in the enzyme activity at various temperature was also observed. The peak activity of the enzyme was observed at the optimum temperature. Further the peak enzyme activity ranged between 40-70^0C suggesting that this thermostable enzyme isolated from *Bacillus pumilus* is advantageous because it increases the reaction rate and it prevents microbial contamination thus contributing to increase in the economical and technical viability of the process (Silva *et al.*, 2005). The optimum temperature (50°C) for xylanase production by *Bacillus pumilus* was similar to some thermophillic fungi such as *Thermomyces lanuginosus* (Purkarthofer *et al.*, 1993) and *Thermosus aurantiaces* (Kalogeris *et al.*, 1998).

Productivity of xylanase can be calculated as the amount or the activity of xylanase produced per unit volume per unit time. For *Bacillus pumilus*, productivity increased with increase in incubation time (7.852 U/ml in 72 hrs), but at 96 hrs of incubation the productivity began to decrease (7.608 U/ml). Similar findings have been reported with *B. licheniformis* where enzyme production reached a maximum level by 72 hrs (Archana and Satyanarayan, 1997, Gupa and Kar, 2008, Widjaja *et al*, 2009). The reduction in xylanase yield after optimum period was probably due to the depletion of nutrient available to microorganism or due to proteolysis by toxic metabolites (Flores *et al.*, 1997).

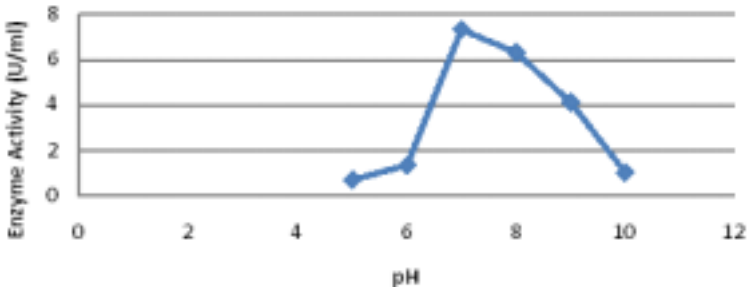

Fig. 3. Xylanase activity at different pH

Effect of pH on xylanase production

At different pH selected for the study, a significant increase in the enzyme activity was recorded from pH 5 (0.710 U/ml) up to pH 7 (7.328 U/ml); thereafter a steady decrease was recorded up to pH 10 (fig.3), pH 7 was found to be optimum for xylanase production making it suitable for bio-bleaching application.

Bandivaelekar and Despande (1994) also reported a pH of 7-8 to be optimum for xylanase production from *Chainia sp.* On contrary a lower pH ranging from 6 – 7 was found to gave maximum xylanase production from *Bacillus sp.* K 1 (Ratanakhanokchai *et al.*, 1999), *Bacillus sp.* C-125 (Honda *et al*., 1985; Rawashdesh *et al*., 2005), *B. subtilis* (Sa-Pereira *et al*., 2002) and *B. stearothermophilus* (Khasin *et al*., 1993).

Effect of substrate concentrations on xylanase production

With increase in xylan concentration from 0.5% to 1.0% a corresponding increase in enzyme activity was recorded (7.302 U/ml, 8.412 U/ml respectively). Further increase in substrate concentration resulted in decreased enzyme activity; suggesting 1% concentration to be optimum for xylanase production. On analyzing the data the difference was found to be statistically significant ($P < 0.05$) (Fig. 4).

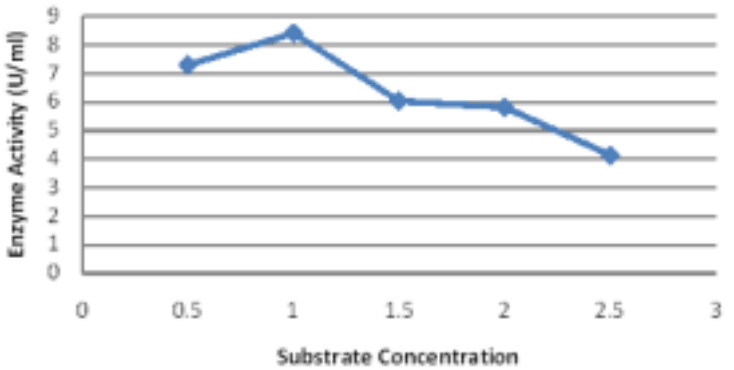

Fig. 4. Induction and repression of xylanase production by *Bacillus pumilus* in medium supplemented with different substrate concentration

Similar observations have been reported in studies conducted previously (Alam *et al*, 1994; Reis *et al*, 2003; Widjaja *et al*, 2009). It appears that low molecular mass fragment of xylan plays a key role in regulation of xylanase biosynthesis. These fragments include xylose, xylobiose etc and the positional isomers (Thomson, 1993; Kulkarni *et al*., 1999).The decrease in the production of xylanase when high amounts of xylan was offered as substrate, may be due to catabolic repression, likewise described for other xylanolytic microorganisms (Kadowaki *et a*l, 1997). Further, use of xylan at low concentrations for maximum xylanase production will be cost effective in large scale industrial processes.

Effect of additional carbon sources on xylanase production (1% w/v)

Among various carbon sources, galactose induced the highest level of xylanase production (7.879 U/ml) followed by maltose (7.125 U/ml) while arabinose gave least productivity (4.254 U/ml) ($P < 0.05$) (Fig. 5).

The decrease in enzymatic activity probably suggests catabolic repression of the sugar in presence of xylan (Rawashdeh *et al.*, 2005). However, an appreciable level of xylanase was also produced in presence of maltose. This may be due to multiple form of xylanase where by one form is produced constitutively in small amounts and the other is induced by substrate xylan (Gessesse and Gashe, 1997).

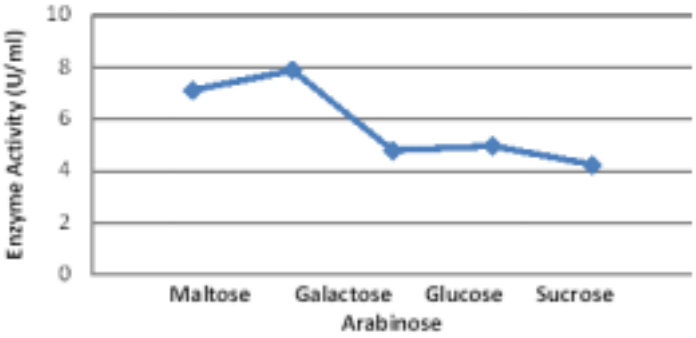

Fig. 5. Production of xylanase influenced by different carbon sources by *Bacillus pumilus*

Xylanase activity decreased at a glucose concentration of 1%. A decrease in the activity of certain catabolic enzymes in the presence of an easily metabolized substrate is called catabolic repression. This effect is caused by glucose (glucose repression). It has been reported previously that glucose addition greatly repressed xylanase activity; the repression being concentration – dependent (Rawashdeh *et al.*, 2005; Bindu *et al.*, 2006),. Perhaps, it is possible that accumulated xylose issued from the hydrolase of xylan lead to this important repression (Senior *et al*., 1989).

Effect of various organic and inorganic nitrogen sources (1%w/v) on xylanase production

Among the various nitrogen sources evaluated $NaNO_3$, beef extract & tryptone gave promising results with beef extract showing maximum xylanolytic activity (7.896 U/ml) ($P > 0.05$) (Fig. 6).

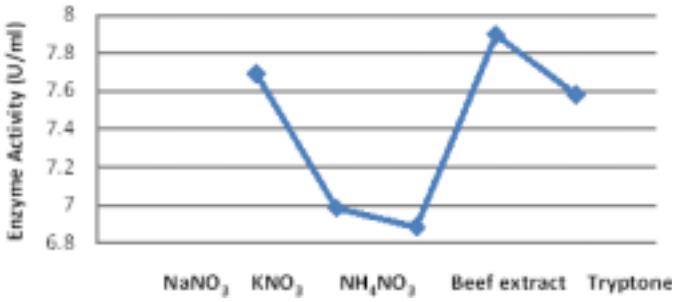

Fig. 6. Effect of different nitrogen sources at 1% concentration by *Bacillus pumilus*

The effect of different nitrogen sources observed is in agreement with the studies of Subramanyian *et al.*, (2001). The positive effects of beef extract, tryptone and $NaNO_3$ on xylanase production by *Bacillus pumilus* could be attributed to the requirement for nitrogen sources during fermentation, whereas NH_4NO_3 and KNO_3 had little effect. The optimal nitrogen sources for xylanase production was diversified depending on the microorganisms tested. It was reported that *Bacillus pumilus* can use low level of tryptone as the best optimal nitrogen source (Amani *et al*,

2007). Gessese, and Mamo, (1999) also reported that there was no significant effect upon addition of peptone or tryptone for xylanase production by *Bacillus sp*. AR-009.

Microorganisms generally degrade xylan only in the absence of other simpler carbohydrates and carbon compounds. The fact that the maximum growth of this *Bacillus sp*. occurs at the highest nitrogen content (beef extract and peptone at 2% w/v) also exemplifies the role of easily fermentable carbon compounds in the medium. Higher amounts of complex nitrogen source for hydrolase-producing bacteria could enhance growth rather than the enzyme production due to the presence of simple sugars (Kelly, 1983).

Also complex nitrogen sources like yeast extract and peptone releases NH_4^+, which would simulate growth (Forage *et al.,* 1985) and at the same time increase enzyme yield because of its inhibition of protease activity around neutral pH (Untrau *et al.,* 1994, Balkrishan *et al.,* 1997). Proteolytic activity in fermentation broth leads to increased degradation of enzymes. Thus the concentration of yeast extract in the medium must be limited to give the required growth (Wanger *et al*., 1995).

Effect of different mineral salts on xylanase production

Among the various mineral salts tested, the culture showed highest xylanase activity when the growth medium was supplemented with $FeCl_3$ (7.948 U/ml) while $CuSO_4$ showed least and KCl showed moderate increase in xylanolytic activity ($P < 0.05$) (Fig. 7).

Effect of metal ions on xylanase activity revealed that enzyme activity was increased by addition of $FeCl_3$ while it was inhibited by $CuSO_4$.These results were not similar to the reported trend for other xylanases (Ratnakhawokchoui *et al.,* 1999, Khandephker and Bhosle, 2006, b).This may be because metal ions not only affect the active site of xylanase but also the non catalytic xylan binding region, which is involved in the efficient hydrolysis of the substrate (Chauvaux *et al*. 1995). However findings are comparable with Khasin *et al*, (1993), Nakamura *et al*, (1993), Xu *et al*, (2008), Ellouze *et al*, (2008), Annamalai *et al*, (2009).

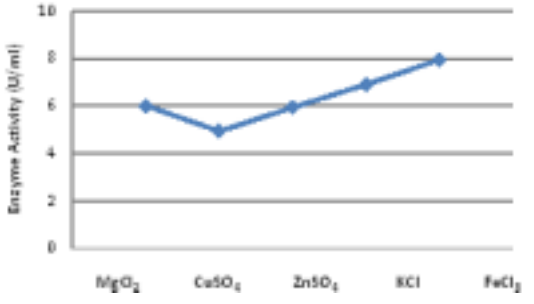

Fig. 7. Effect of minerals additives on xylanase production by *Bacillus pumilus*

CONCLUSION

The results obtained from submerged culture indicate that significant improvement of xylanase production by *Bacillus pumilus* strain could be through selective use of nutrients and growth conditions. Hyper production of xylanase by *Bacillus* species reported earlier is confirmed in the present study. Infact the maximal enzymatic production obtained after 3 days incubation corresponding to 7.852U/ml was higher than the levels cited in the literature. Therefore, xylanase from *Bacillus pumilus* produced under optimized production conditions exhibits favorable potential to the paper and pulp industry.

Further aspect of this work can be performed to reduce the substrate cost by using agricultural residues in solid state fermentation. Purification and characterization of the target protein, protein sequencing and *insilco* modeling can also be done.

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