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Utilization of microwave irradiated rice straw as a substrate for cost effective production of cellulase and xylanase from a potential fungus *Myceliopthora thermophila* SH1 isolated from hot spring of Northern Himalayans

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ABSTRACT

Cellulase and xylanase production from rice straw using M.thermophila SH1 was assessed. The waste was dried, grinded to mesh size of 2.0mm and microwave pretreated. The powdered waste was than used as a substrate. Fermentation was carried out in flasks containing pretreated and untreated rice straw, vogel;s medium, cultured at 50^0C initially for 8 days to verify cellulase production and for 5 days for xylanase production. The results showed that M.thermophila SH1 produced the highest amount of cellulase i.e. 43.07U/gds and xylanase of 281.07U/gds using pretreated rice straw under SSF. An increased in enzyme activity was observed in solid state fermentation over submerged fermentation.

Key words: Thermophilic fungus, cellulase, xylanase, hot springs, solid state fermentation.

INTRODUCTION

Cellulase and xylanase have immense potential in many industries viz. detergents, clarification of fruit juice, bakery products, biopulping, to improve silage (Bhat, 2000; Beg et al., 2001). Cellulase enzyme hydrolyzes β-1,4-glucosidic bonds in cellulose polymer to release glucose units. Biological degradation of cellulose involves action of three enzymes namely endo-β-1,4-glucanase, exo-β-1,4-glucanase and β-glucosidase. Endo-β-1,4 glucanase or carboxymethyl cellulose (CMCase), hydrolyzed cellulose in random fashion producing oligos and reducing polymer length, while exo-β-1,4-glucanase (cellobiohydrolyse) cleave cellobiosyl residue from the non-reducing end of cellulose chain (Kaushal etal., 2012). Cellulases are industrially important enzymes that are sold in large volumes at very high cost for use in different industrial applications *viz.* in starch processing, animal feed production, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper industry and textile industry (Ogel et al., 2001). There is a growing market for cellulase in the field of detergents and saccharification of agriculture waste for bioethanol technology.

Xylanase enzymes catalyze hydrolysis of xylan, usually associated with cellulose and lignocelluloses components. Several enzymes are involved in the hydrolysis of xylan polymers of which the most important is the endo-1,4-β xylanase (EC 3.2.1.8) (Sa-Paveia et al., 2003). It has received considerable interest due to its significant applications in various industrial processes such as paper and pulp industries, food, feed, waste treatment, fuel and chemical production (Chantasingh et al., 2006).

Terrestrial geothermal areas are located in various regions of our planet, these sites includes thermal springs, fumaroles and geysers are the common places or are rich sources of thermophilic microorganisms (Johnson et al., 2003). A number of extreme thermophiles or hyperthermophiles within the domain of bacteria, fungus and archea have been isolated from terrestrial hot springs (Gosh and Gosh, 1992). First time existence of microorganisms with optimal growth temperature has been reported by Brock (1967). One of the most attractive attribute of thermophiles is that they produce enzyme capable of catalyzing biochemical reactions at temperature higher than those of mesophilic microorganisms (Demirijian et al., 2001). Thus interest in enzymes produced from theses microorganisms (hot spring) has increased worldwide owing to their potential commercial application (Lynd et al., 2005).

Thermophilic fungi are naturally excellent protein secretors and can produce enzymes in industrially feasible amounts. Owing to the increased biotechnological importance of thermostable cellulase and xylanase, many thermophilic microorganisms isolated from soil have been examined for production of these hydrolytic enzymes (Topkas et al., 2003).

Solid state fermentation (SSF) has been considered as a promising mode for production of enzymes. Among the microorganisms, fungi are considered to be the most adapted to SSF because their hyphae can grow on particle surface as well as penetrate deep into the inner particle spaces and thereby colonizing solid substrates effectively (Pandey, 2002). The suitable substrate chosen for micro-organism cultivation aiming the enzymatic biosynthesis depends on a series of factors which include cost and usage viability. The use of rice straw can be a inexpensive carbon source for large scale utilization (Yang et al., 2006). Pretreatment alter or remove structural and compositional impediments to hydrolysis and subsequent degradation processes in order to enhance digestibility (Moiser et al., 2005). Microwave irradiated pretreatment was chosen in solid state fermentation study because of the reasons *viz.* highly effective, physical method and pollution free approach as no discharge/ toxic byproducts are generated.

MATERIALS AND METHODS

Microorganism
Myceliopthora thermophila SH1 sequence had been deposited in Genbank database and provided with an accession number NCBI-JX124712 (Sharma et al., 2013). The stock culture was maintained on agar slants at 5°C.

Enzyme production studies
Submerged fermentation:
The vogel's medium supplemented with 1% of cellulose/xylose with pH adusted to 5.5 before autoclaving at 121^0C for 15 min. Then the 100ml medium was inoculated with 10 ml of spore suspension (1×10^7 spores/ml). The flasks were incubated at 50°C on rotatory shaker (120 rpm) for 5 days. After incubation the filtrate was centrifuged at 10,000 rpm for 15 min at 4°C. The clear suspension was used for enzyme assays.

Enzyme assay
Cellulase assay: The activities of total cellulose i.e. filter paper activity, endoglucanase and β-glucosidase was determined using standard methods of FPase and CMCase (Reese and Mendel, 1963) and β-glucosidase (Berghem, 1973).

Xylanase assay: Similarly the activity of xylanase in the culture filtrate was determined by Millers method (Miller, 1959).

One international unit (IU) of enzyme activity represents µmoles of xylose/glucose/p-nitrophenol released/min/ml of enzyme.

Protein assay:
Protein content of culture filtrate was also determined by Folin-Ciocalteu reagent using Bovine Serum Albumin (BSA) standard (Lowry et al., 1951).

Since *M. thermophila* SH1 depicted good activity of cellulase and xylanase enzymes under SmF. Therefore, these experiment was planned in solid state fermentation (SSF) mode by optimizing for best condition i.e. moistening

agent obtained in SmF. However agriculture waste has been replaced with commercially available substrates i.e. cellulose and xylan as carbon source to get escalate enzyme yield using inexpensive waste material for cost effective production.

Submerged fermentation
Optimization of moistening agent/media
The effect of different moistening agents i.e. Vogel's medium, Synthetic medium, Czapeks medium and Basal salt medium on cellulase and xylanase production was tested at incubation temperature of 50^0C. The supernatant was collected and enzyme activity was assessed.

Solid state fermentation
Microwave assisted pretreatment of biomass/ substrate preparation
Rice straw (*Oryza sativa)* an inexpensive agricultural waste that has been generated in bulk can be utilized as cellulosic substrate for the production of cellulase and xylanase enzymes. The feedstock was oven dried to remove moisture and was milled to reduce particle size of pass a 2mm sieve and stored in sealed plastic bag at room temperature. For microwave pretreatment. 100 g of agricultural residue was taken in a beaker and was microwave (Godrej make) irradiated at 250 V and 50 Hz for 2 min.

Production of enzyme
M.thermophila SH1 was cultivated in 250ml Erlenmeyer flask containing 5g of substrate with addition of vogel's medium (Vogel, 1956) as a moistening agent at four different concentrations i.e. 20, 40. 60 and 80 ml to maintain substrate: moisture ratio of 1:2, 1:4, 1:6 and 1:8 respectively as moisture plays vital role in enzyme production under SSF. The vogel's medium was prepared with composition i.e. (g/L) trisodium citrate (0.5), KH_2PO_4 (0.5), NH_4NO_3 (0.2), $(NH_4)_2SO_4$ (0.4), $MgSO_4$ (0.02), peptone (0.1), yeast extract (0.2), glucose (0.2), agar (2.5), pH 5.5. The substrate was inoculated with *M.thermophila* SH1 and then incubated at 50°C for 8 days.

Enzyme extraction:
To 5g of untreated and treated biomass of each set 50 ml of phosphate buffer (0.1M, pH 6.9) with 0.1% Tween-80 was added in 250 ml of Erlenmeyer's flask. The contents were kept under agitation at 120 rpm for 60 min and then filtered through muslin cloth. The process was repeated twice using 25ml of phosphate buffer every time and thus making final volume to 100 ml. After the filtration, contents were centrifuged at 5,4000 rpm for 10 min at 4^0C (Bollag and Edelstein, 1993). The cell free supernatant was used as the source of crude enzyme preparation and then used for enzyme assays.

RESULTS

In the present study, we attempted the production of cellulase and xylanase from microwave pretreated biomass an agroresidue from *M.thermophila* SH1 isolated from hot springs of Himachal Pradesh under different mode of fermentations (Sharma et al., 2013).

Submerged fermentation
Media optimization for Cellulase and xylanase production
Optimization of moistening agent/medium was done for increasing titers of enzymes. The enhanced cellulase production on vogel's medium was observed on 8^{th} day of incubation i.e. 0.844 IU/ml and xylanase activity of 9.20 IU/ml after 5 days **(Fig.1)**. Enhanced enzyme production using defined medium was due to the presence of nitrogen, carbohydrate and other compounds in adequate quantity that could be utilized easily by the growing fungus thus enhancing the cell ability to produce enzymes. Due to the meager production of cellulase and xylanase by thermophilic fungus under submerged fermentation, the production of cellulase and xylanase was shifted submerged over to solid state mode of fermentation to enhance the enzyme titers. Among the lignocellulosic residues as carbon source rice straw was chosen as a cost-effective substrate for enzymes production and microwave pretreatment was given to it.

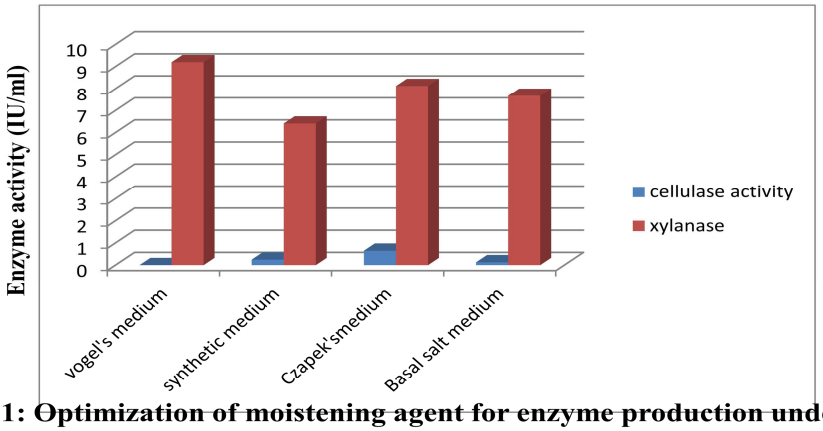

Fig 1: Optimization of moistening agent for enzyme production under SmF

Solid state fermentation

Effect of moisture level on cellulase and xylanase production

Moisture content is a critical factor of a cell growth and enzyme production under SSF, which determine the outcome of the process. As shown in Fig 3. The optimum initial moisture level was 1:6 for enzymes production by *M.thermophila* SH1 on pretreated rice straw i.e. 43.07 U/g as compared to untreated biomass i.e. 33.54 U/g of cellulase and it declined with further increase in substrate to moisture ratio i.e. at 1:8 (substrate: moisture ratio) with 24.32 U/g of cellulase production in pretreated biomass **(Fig. 2 and Plate 1)**. Similarly **Fig. 3 and Plate 2** shows that a maximum activity of xylanase was recorded at 1:6 (substrate: moisture) level after 5 days incubation at 50°C using pretreated rice straw as a substrate and lesser xylanase activity at moisture level 1:8 i.e. 201.82 U/g due to increase in moisture level. Pretreatment of lignocellulosic biomass is a prerequisite to increase accessibility of cellulose as well as to remove structural and compositional implements to hydrolysis (Sharma et al., 2006). Microwave irradiated pretreatment of rice straw was chosen in the present study because this method had been found a very effective physical method to open up the structure of cellulose alongwith an eco-friendly approach as no discharge/toxic byproducts are generated in nature during irradiation. Action mechanism for microwave is when crystalline region is placed between electromagnetic field it will polarize generating a charge on crystalline interphase and therefore, increase the amorphous region of hemicellulose softening.

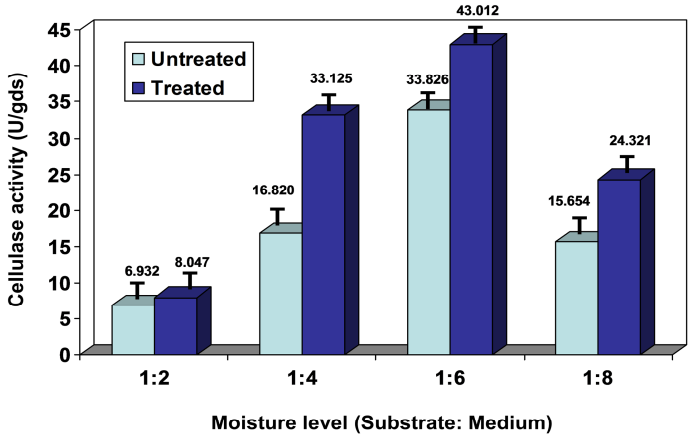

Fig 2. Comparison of cellulase activity at different moisture level between untreated and microwave treated rice straw from *M.thermophila* SH1under SSF

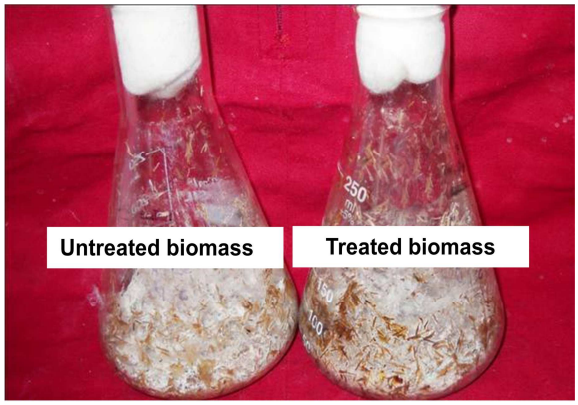

Plate 1: Effect of moisture on cellulase production under solid state fermentation.

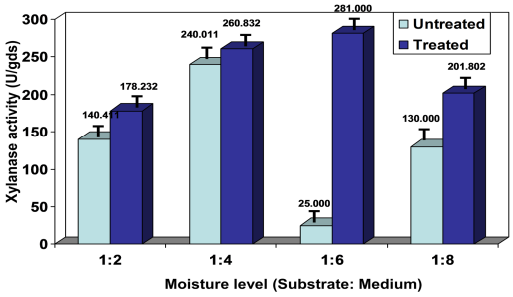

Fig 3. Comparison of xylanase activity at different moisture level between untreated and microwave treated rice straw from *M.thermophila* SH1 under SSF

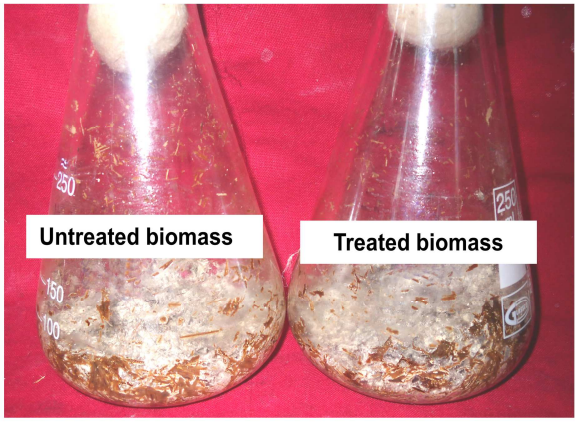

Plate 2: Effect of moisture on xylanase production under solid state fermentation

Comparison of SmF and SSF

Fig 4 reveals a comparision between SSF and SmF on cellulase and xylanase producing abilities of *M. thermophila* SH1. The cellulase activity of *M. thermophila* SH1 under SSF condition was 1.71 IU/ml which was higher than 0.84 IU/ml SmF condition. Similarly xylanase activity *M. thermophila* SH1 was increased from 9.20 IU/ml to 10.05 IU/ml under SSF. SSF has many advantages as compared to the SmF viz. simple technology, high volumetric productivity reduced downstream processing costs, low water requirement and high enzyme concentration. SSF was predominantly useful for enzyme production by fungi might be the enhanced physiological processes in cell adhesion or biofilm formation (Dutt and Kumar, 2012).

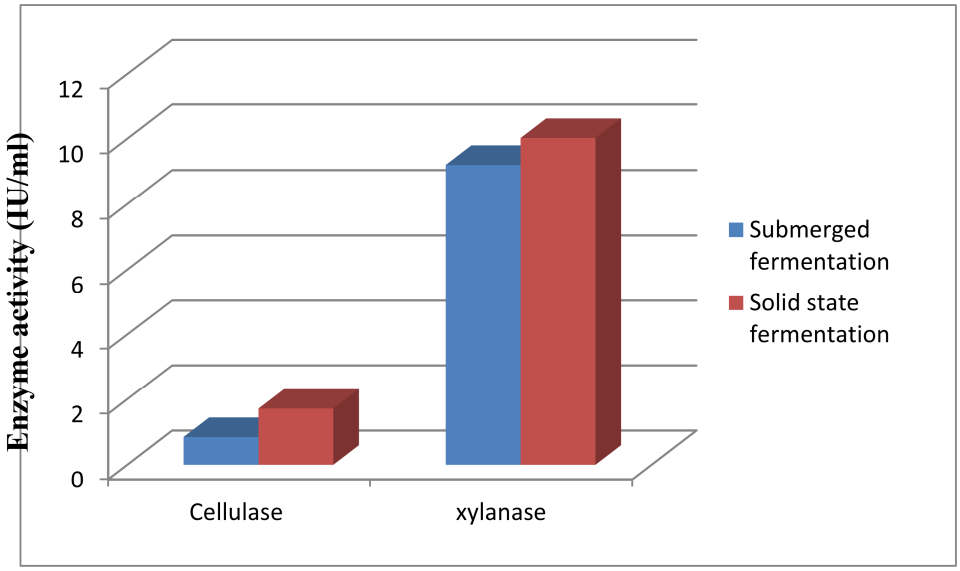

Fig. 4 : Comparison of enzyme production under SmF and SSF

CONCLUSION

Rice straw seems to be a suitable substrate as cellulase and xylanase are inducible enzymes and cellulose and xylan present in rice straw act as good inducers for enzyme production. Xylanase and Cellulase activity was obtained in solid state fermentation of rice straw by fungus under the optimized condition. The present work should that microwave pretreated rice straw was a suitable source of cellulase and xylanase production by *M.thermophila* SH1 at an incubation temperature of 50^0C, cellulase activity achieved 43.07U/gds after 8 days of incubation and xylanase activity of 281.07 U/gds after 5 days of incubation higher than the enzyme production under submerged fermentation. SSF has emerged as a preferable mode for higher yield of cellulase as well as xylanase by using agricultural lignocellulosic waste. To reduce the cost of enzyme production, lignocellulosic substrate is an attractive approach rather than opting for expensive substrate for enzyme production.

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