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RESEARCH ARTICLE

PRODUCTION AND OPTIMIZATION OF LIGNOCELLULASES BY *ASPERGILLUS AWAMORI* IN SUBMERGED FERMENTATION

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ABSTRACT

Aspergillus awamori is an efficient producer of many hydrolytic enzymes, has not been exploited commercially and for enzyme production. Cellulases, Xylanases and laccases thought to be of great significance for several industries namely paper, pharmaceuticals, food, feed etc. in addition to better utilization of lignocellulosic biomass. The present investigation was aimed to produce several extracellular enzymes by *Aspergillus awamori* and the Molecular characterization of the isolate by18S rRNA sequencing. Commercially available agricultural waste solid substrates were used as cellulosic substrates for enzyme production. Experiments were conducted to optimize the production of lignocellulases. The maximum enzyme activity was achieved on 6th day of incubation at 30^{O} c temperature with the pH 6.0 and by using Lactose 1% w/v as Carbon Source, tryptone, yeast extract 1% w/v as Nitrogen Source. The present study reveals that the cellulolytic properties of *Aspergillus awamori* tested and optimized culture conditions *maximizing* lignocellulolytic enzymes production.

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INTRODUCTION

Lignocellulose is in general the raw material for the paper industry, besides that, it is a potential source for production of bio fuels, bio-fertilizers, animal feed and chemicals (Okeke and Lu, 2011). Exploitation of this renewable resource requires pretreatment of the material either biologically or chemically, cellulases are used in later stages. Research has started exploring the mechanisms of microbial cellulose production that paved ways for development of technologies that would significantly increase the production and applications of cellulose degrading enzymes. In view of the above, the proposed study was aimed to produce lignocellulolytic enzymes like cellulase, xylanase, β-D-glucanase, laccase by *Aspergillus awamori*. The fungal strain was used in this study is *Aspergillus awamori*, most probably it was found to be as plant pathogen especially in monocot plants cause black mold diseases, invades the plant cell walls, release hydrolytic enzymes and cause infection at mesoderm region. Spores of this fungus are very common in the air and soil. The current study is taken up with two essential objectives of isolation of highly efficient lignocellulase producing source organism, production of lignocellulases in higher quantities from the isolated source through Submerged fermentation, and optimization of fermentation conditions.

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MATERIALS AND METHODS

Collection and Analysis of Solid Substrates

Locally available lignocelluloses Paddy straw, Wheat husk, Groundnut haulms were used in the present study. substrates were individually grounded and sieved through a 2mm screen, for uniform particle size. Test organism used in this present study is *Aspergillus awamori,* isolated form rotten paddy straw collected from Sri Venkateswara Gosamrakshana Shala, Tirupati. The isolate was screened by qualitative screening for its multiple enzyme producing ability (Charitha Devi and Sunil Kumar, 2012). Molecular identification of the isolate was done by sequencing 18S rRNA and taxonomic or phylogenetic analysis was performed, the sequence was deposited in Genbank Database with allotted Accession No KM660725.1.

Initial production of lignocellulases by Submerged Fermentation (SmF)

Quantitative Screening of lignocellulosic producing ability of *Aspergillus awamori* was determined by submerged fermentation under non optimised conditions. The spore suspension (0.5 ml) was inoculated into 50 ml of sterilized production medium (1%, v/v) in 250 ml Erlenmeyer flask maintaining sterile conditions. The uniform spore count ($\sim 16X10^4/mm^3$) was maintained to each flask for enzyme production. The flasks were incubated at 30^{o}C with 120 rev

min^{-1}. At intervals of 2nd, 4th, 6th and 8th day fermented culture broth (50 ml) was collected and filtered through the What man filter paper, 4 ml of the filtrate centrifuged at 8000 rpm/15 min at 4°C and the filtrate was used for enzyme assays.

Enzyme assays

Enzyme assays were performed by using specific synthetic substrates based on the standard methods like assay of endoglucanse and exoglucanase activity: Ghosh,1987; Assay of β-D-Glucosidase activity: Herr (1979); Assay of Xylanase activity: Bailey and Lumsden (1998); Assay of laccase activity: (Dass *et al.,* 1997); estimation of Extracellular Protein Content: Lowry *et al.* (1951).

Optimization of lignocellulolytic enzyme production in submerged fermentation (SmF)

The cultural conditions were optimized for higher yield of lignocellulase enzymes. For the initial optimization of the medium, the traditional method of "one variable at a time approach" was used by changing one component at a time while keeping the others at their original level. The cultural conditions like pH, temperature, carbon and nitrogen sources were optimized the independent variables like pH ranging from 4.0 to 7.0, temperature levels at 25°C, 30oC, 35°C and 40°C, incubation time 2 to 10 days were studied. Different carbon sources i.e., Sucrose, Glucose and Lactose and different nitrogen sources such as urea, Tryptone and yeast extract were added individually at 1% (w/v) to the medium to optimize the nutritional parameters.

RESULTS AND DISCUSSION

Several Fungal species with appropriate cellulolytic activity were isolated from collected samples. Based on the morphological characteristics like colour of the colony, vegetative and reproductive structures; certain growth pattern studies; and 18S rRNA sequencing the organism has been identified as *Aspergillus awamori*. consensus Bootstrapped phylogenetic tree was constructed by the neighbor joining tree method, constructed using Clustal X v. 2.1 with 1000 replicates and 111 random odd numbers; by using MEGA 6.0 (Tamura *et al.,* 2007) to evaluate the evolutionary taxa of our strain of interest (Felsenstein, 1985). The consensus sequence database of *Aspergillus awamori* 18 S rRNA was deposited in NCBI Genbank with an accession number KM660725.1. (Fig.1.).

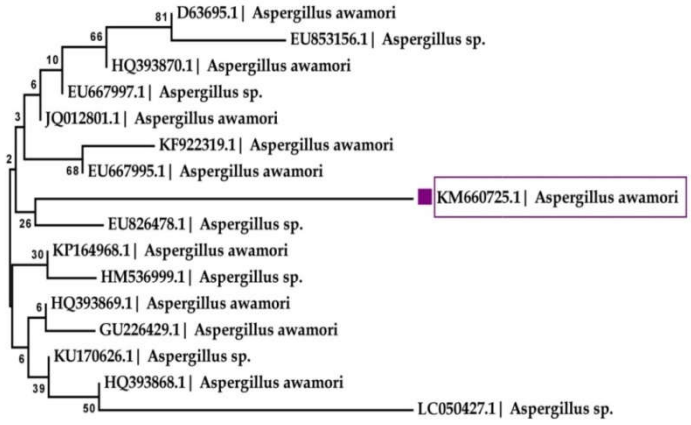
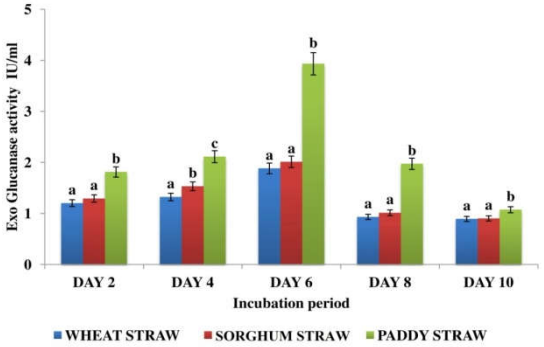


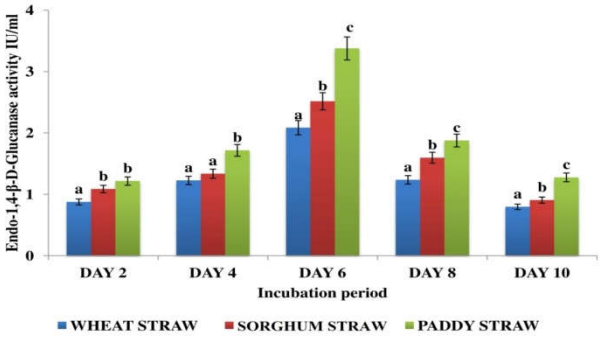
Fig. 1. Bootstrapped neighbor joining phylogenetic tree of *Aspergillus awamori* with Genbank ACC No. *KM660725.1*

All the fermentation studies were performed in triplicate, the mean and standard deviation values were taken into consideration for constructing bar diagrams. *Aspergillus awamori* showed maximum lignocelluase activity on the 6th day of incubation with paddy straw as best utilizing cellulosic substrates. Initially non optimized cultural conditions were maintained to detect the quantitative enzyme producing ability. The enzyme activities were measured as exoglucanase activity of 3.93 IU/ml, endoglucanase activity of 3.37IU/ml , β-glucosidases activity of 2.6 IU/ml, Xylanase activity of 6.93 IU/ml, Laccase activity of 2.08 IU/ml and total protein content 6.38 mg/ml of culture filtrate, followed by incubation with sorghum straw showed acceptable enzyme activities of exoglucanase activity of 2.0 IU/ml, endoglucanase activity 2.51IU/ml, β-glucosidases activity 1.2 IU/ml, xylanase activity of 5.01 IU/ml, laccase activity of 1.22 IU/ml and total protein content 5.55 mg/ml on 6th day of incubation. (Fig.2., 3., 4., 5., 6. and 7).



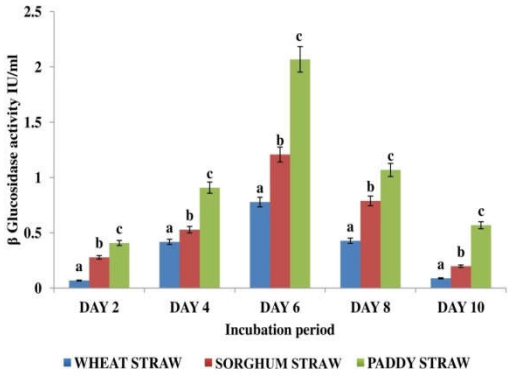
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 2. Initial production of exoglucanse by *A. awamori* in SmF



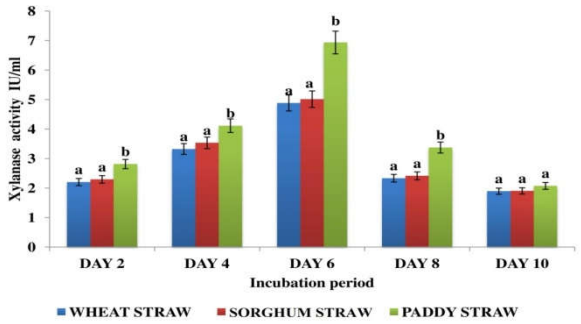
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 3. Initial production of endoglucanse by *A. awamori* in SmF



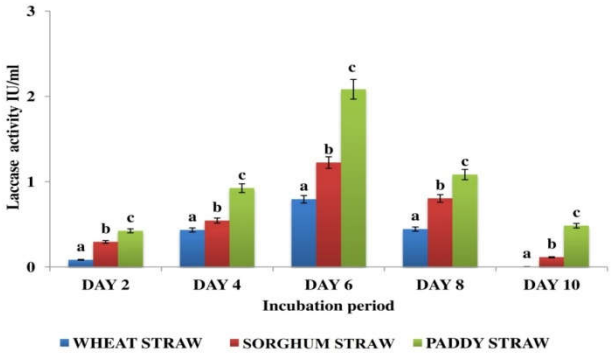
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 4. Initial production β-glucosidases by *A. awamori* in SmF



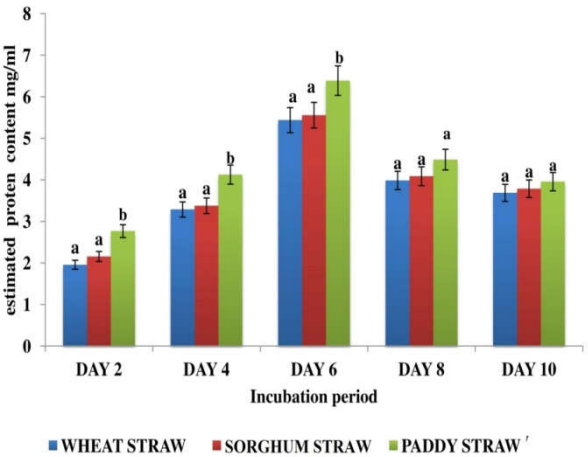
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 5. Initial production of xylanase by *A. awamori* in SmF



Bars with same superscripts are not significant from each other at p<0.05.\

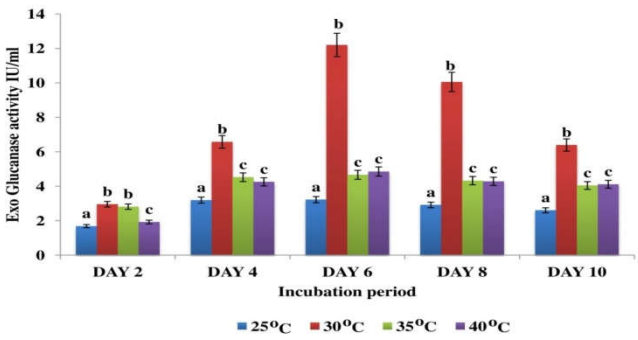
Fig. 6. Initial production of laccase by *A. awamori* in SmF



Bars with same superscripts are not significant from each other at p<0.05.

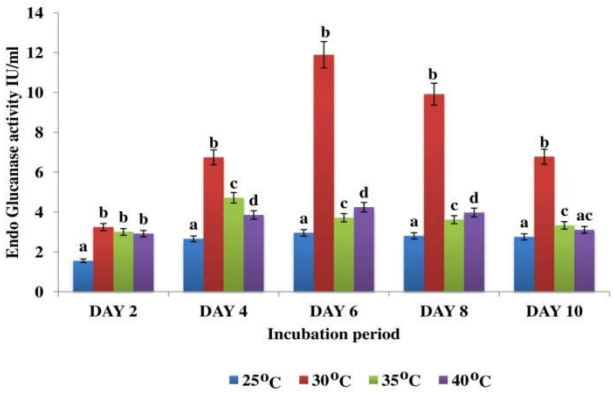
Fig. 7. Total protein content estimated by *A. awamori* in SmF

The physical factors like incubation temperature and initial medium pH and nutritional factors such as carbon and nitrogen sources were optimized for production of lignocellulolytic enzymes under submerged liquid fermentation by *A.awamori.* The effect of incubation temperature on production of lignocellulolytic enzymes found to be higher at 30°C as compared to remaining incubation temperatures. The maximum exoglucanase activity of 12.21 IU/ml, endoglucanase activity 11.9 IU/ml, β-glucosidases activity 3.01 IU/ml, xylanase activity of 12.9 IU/ml, laccase activity of 2.44 IU/ml and total protein content of 35.37 mg/ml was measured on 6th day of incubation at 30°C incubation temperature with paddy straw as lignocellulosic source of substrate and the all enzyme activities were gradually decreased as temperature increased (Fig. 8., 9., 10., 11., 12. And 13.).



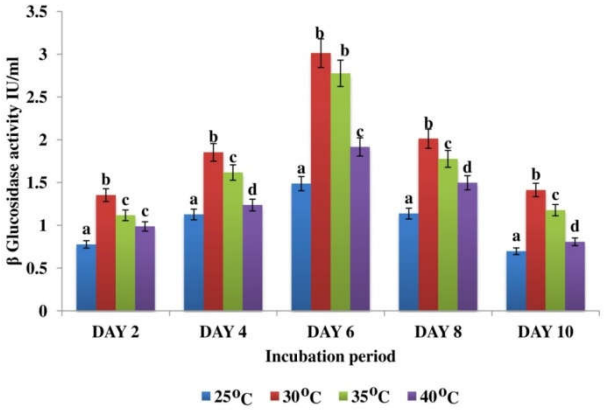
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 8. Effect of temperature on exoglucanse production by *A. awamori* in SmF



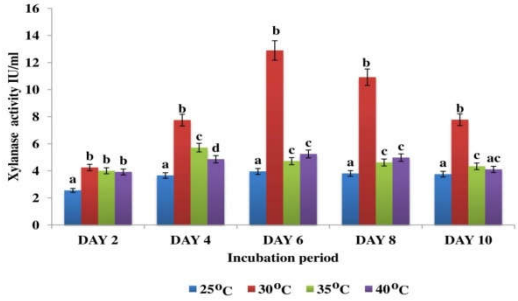
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 9. Effect of temperature on endoglucanse production by *A. awamori* in SmF



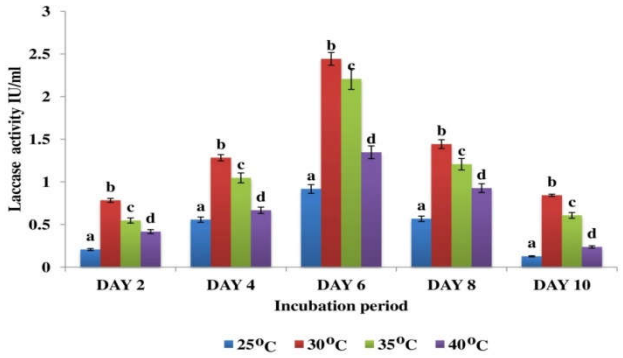
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 10. Effect of temperature on β glucosidase production by *A. awamori* in SmF



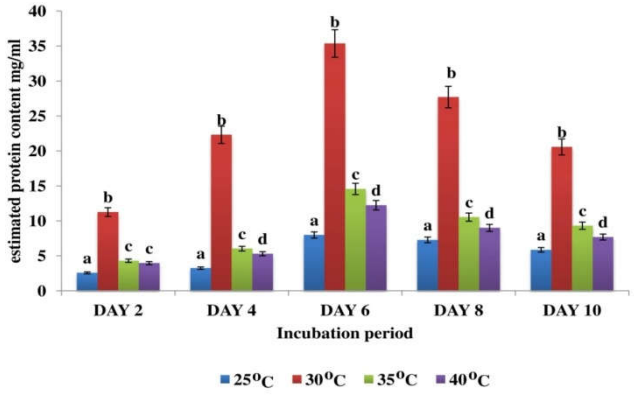
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 11. Effect of temperature on xylanase production by *A. awamori* in SmF



Bars with same superscripts are not significant from each other at p<0.05.

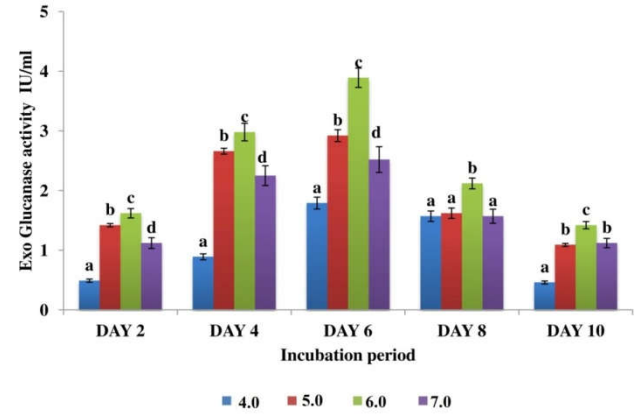
Fig. 12. Effect of temperature on laccase production by *A. awamori* in SmF



Bars with same superscripts are not significant from each other at p<0.05.

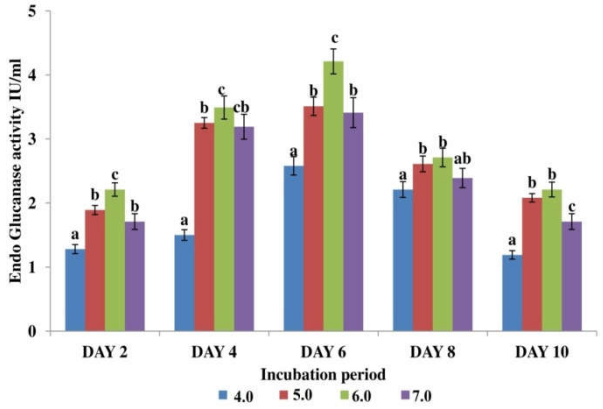
Fig. 13. Effect of temperature on total protein content by *A. awamori* in SmF

Optimum production of cellulolytic enzymes in shake-flask cultures at 30°C has been reported for several fungi (*Penicilliumcitrinum* – Olutiola, 1976; *A. niger* VKMF-2092 - Kerns *et al.* 1987; *T.reesei* Rut C 30 – Szengyel, 2000; *Mucorcircinelloides* –Badal, 2004; Szijarto *et al.* 2004; *Trichoderma, A.glaucus* XC9 - Chang *et al.,* 2006). Maximum enzyme activity was recorded at pH 6.0 and the activity was measured as exoglucanase activity of 2.92 IU/ml, endoglucanase activity 3.51IU/ml, β-glucosidases activity 3.01 IU/ml, xylanase activity of 6.21 IU/ml, laccase activity of 3.32 IU/ml and total protein content 11.54 mg/ml on 6th day of incubation at 30^0 c incubation temperature with paddy straw as lignocellulosic source of substrate.



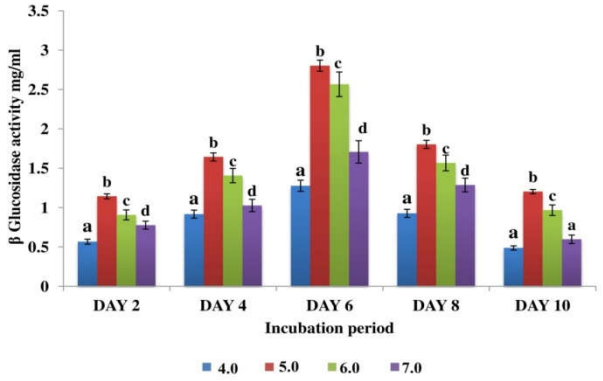
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 14. Effect of pH on exoglucanse production by *A. awamori* in SmF.



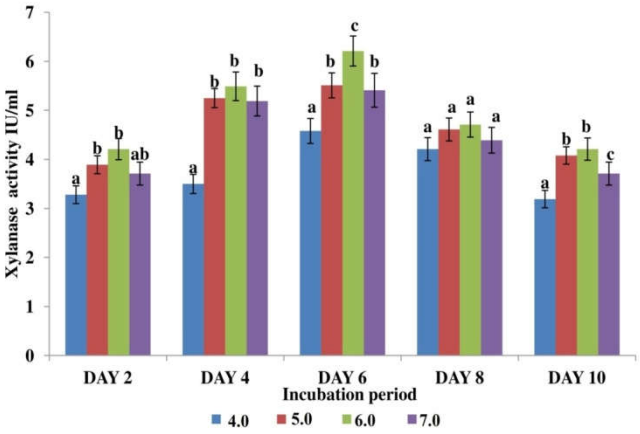
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 15. Effect of pH on endoglucanse production by *A. awamori* in SmF.



Bars with same superscripts are not significant from each other at p<0.05.

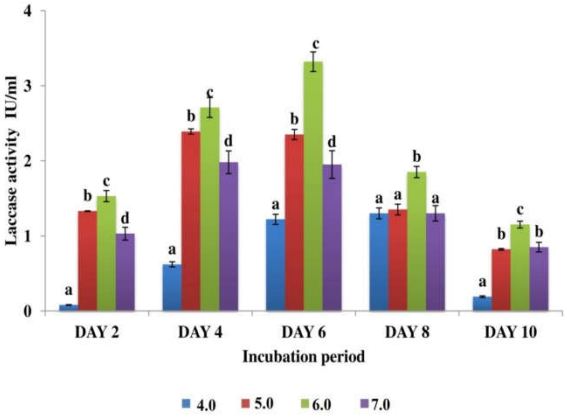
Fig. 16. Effect of pH on β-glucosidase production by *A. awamori* in SmF



Bars with same superscripts are not significant from each other at p<0.05.

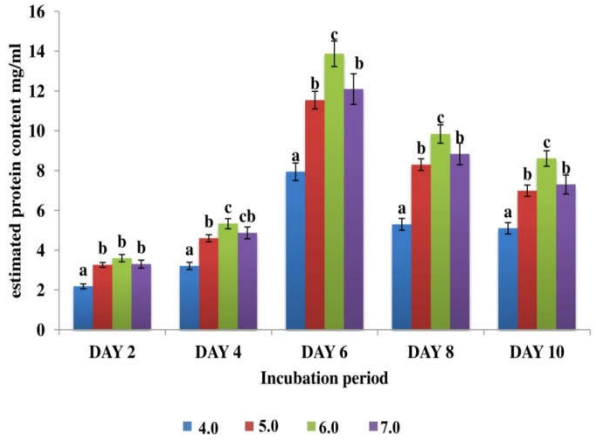
Fig. 17. Effect of pH on xylanase production by *A. awamori* in SmF.

(Fig. 14., 15., 16., 17., 18., and 19.). The optimum initial medium pH for *A. awamori* in the present study was found to be 6.0. An optimum pH range of 5.5 – 6.0 was reported for maximum production of enzymes by *A. glaucus*XC9 (Chang *et al.,* 2006), *A. niger* (Johansson and Reczey, 1998), and *T. reesei* (Szengyel, 2000). On the other hand, optimum initial pH of 7 was reported for various cellulolytic organisms such as *A.fumigatus*, Neurosporacrassa and *Sporotrichum thermophile* (Stewart and Parry, 1981; Eberhart *et al.,* 1977; Coutts and Smith,1976). The optimal pH in the acid range was reported for cellulolytic fungi such as *T.reesi* strain QM-9414 pH 3.5



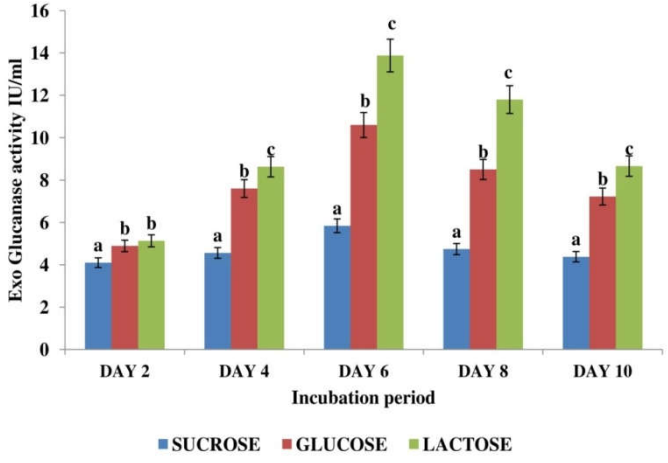
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 18. Effect of pH on laccase production by *A. awamori* in SmF.



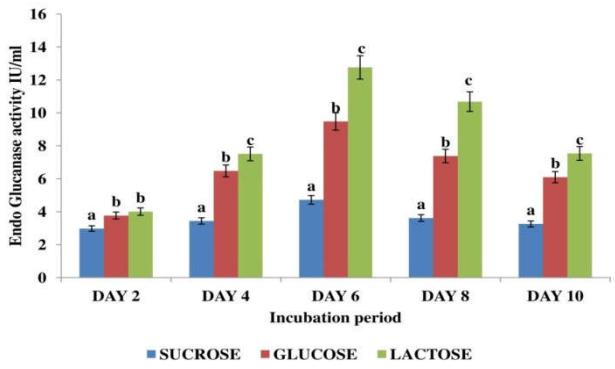
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 19. Effect of pH on total protein content by *A. awamori* in SmF.



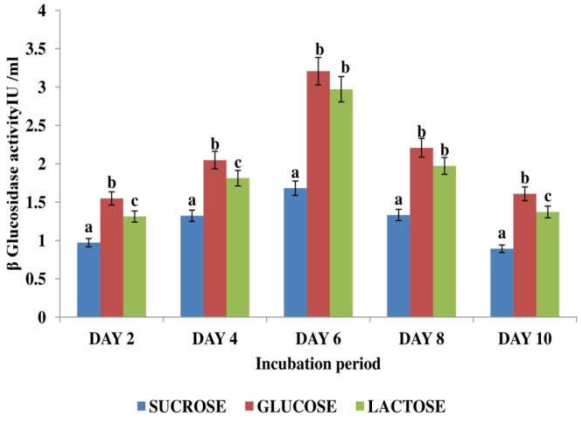
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 20. Effect of carbon sources on exoglucanse production by *A. awamori* in SmF.



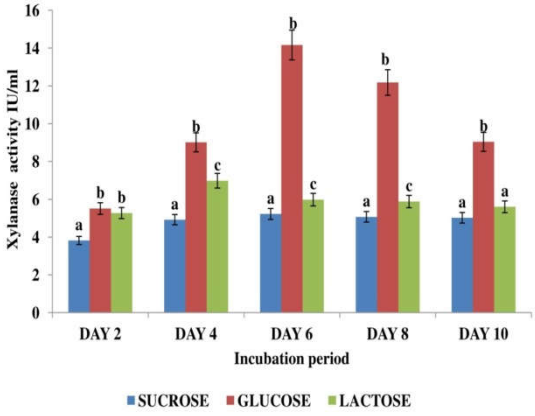
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 21. Effect of carbon sources on endoglucanse production by *A. awamori* in SmF



Bars with same superscripts are not significant from each other at p<0.05.

Fig. 22. Effect of carbon sources on β glucosidase production by *A. awamori* in SmF.

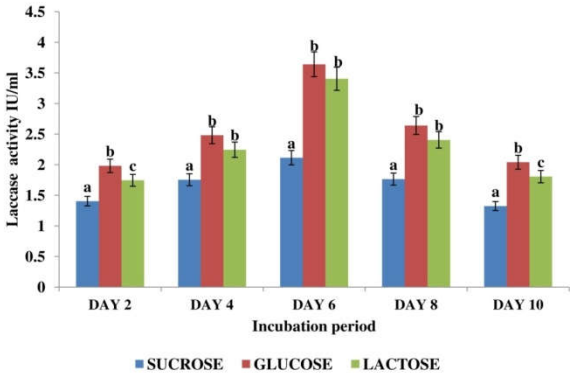


Bars with same superscripts are not significant from each other at p<0.05.

Fig. 23. Effect of carbon sources on xylanase production by *A. awamori* in SmF.

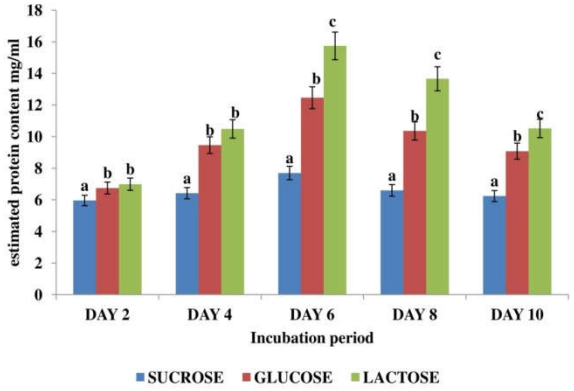
(Krishna *et al.,* 1999), *T.reesi* strain MQ 6a pH 2.8 (Sternberg and Mandels 1979) and *A.terreus* pH 5 (Oikawa *et al.,* 1998; D'Souza and Volfova, 1982). Among the carbon sources amended in the fermentation broth glucose supplementation yielded maximum enzyme activities of β-glucosidases 3.2 IU/ml, xylanase 14.61 IU/ml and laccase activity of 3.64 IU/ml, whereas the supplementation of lactose showed maximum enzyme activities of exoglucanase13.88 IU/ml, endoglucanase 12.76 IU/ml and total protein content of 15.74 mg/ml on 6th day of incubation at initial pH 6.0, incubation temperature 30^{o}C respectively with paddy straw as lignocellulosic source of substrate.

(Fig. 20., 21., 22., 23., 24. and 25.). The tested carbon sources like glucose, sucrose and lactose at different concentration induced cellulase production. CMC found to be significant for inducing cellulase production Olutiola (1976) reported similar result by *Penicillium citrinum.* Lactose and CMC were also reported to be optimal inducers of cellulase production by *Myceliophthora thermophila* D-14. Highest cellulases production by *Schizophyllum commune* was recorded with thiocellobiose, but not with CMC, cellobiose and avicel (Rho *et al.,* 1982). In contrary, amorphous celluloses stimulated higher yields of cellulase from *A. fumigates* (Stewart and



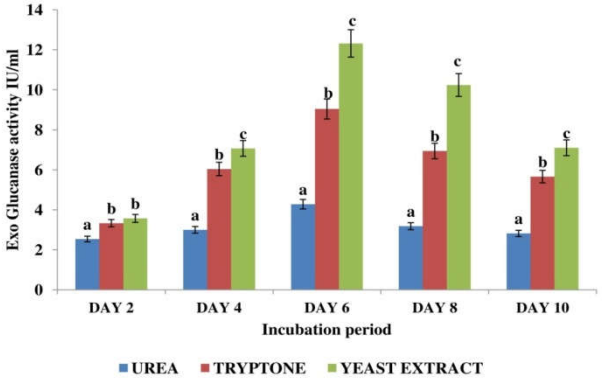
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 24. Effect of carbon sources on laccase production by *A. awamori* in SmF



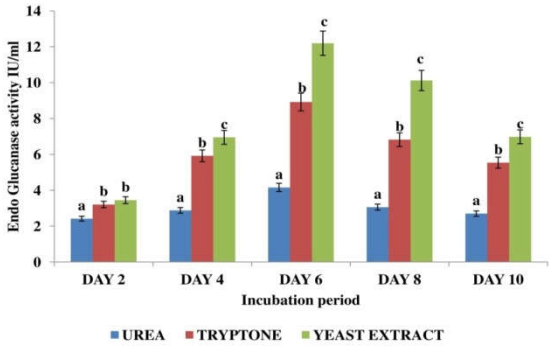
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 25. Effect of carbon sources on total protein content by *A. awamori* in SmF.



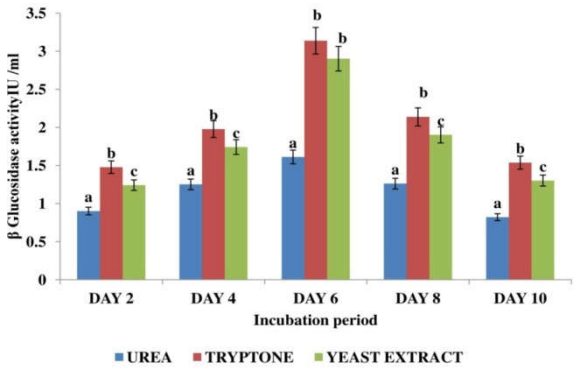
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 26. Effect of nitrogen sources on exoglucanse production by *A. awamori* in SmF.



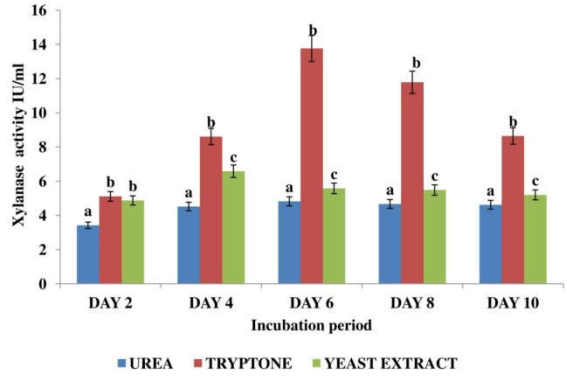
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 27. Effect of nitrogen sources on endoglucanse production by *A. awamori* in SmF.



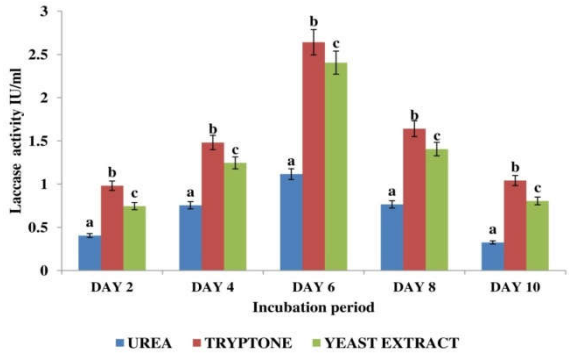
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 28. Effect of nitrogen sources on β-glucosidase production by *A. awamori* in SmF.



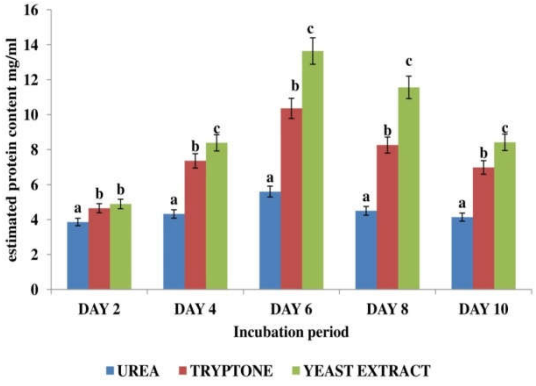
Bars with same superscripts are not significant from each other at p<0.05.

Fig. 29. Effect of nitrogen sources on xylanase production by*A. awamori* in SmF.



Bars with same superscripts are not significant from each other at p<0.05.

Fig. 30. Effect of nitrogen sources on laccase production by *A. awamori* in SmF.



Bars with same superscripts are not significant from each other at p<0.05.

Fig. 31. Effect of nitrogen sources on total protein content by *A. awamori* in SmF.

Parry, 1981), *Thermomonosporafusca* (Spiridonov and Wilson, 1998) and *T.harzianum* (Rousses and Raimbault, 1982). The substrate inhibition of cellulolytic enzymes production by *A.japonicus, A.niger* was reported by supplementation of glucose at higher concentration to the media (Sanyal *et al.,* 1988; Gokhale *et al.,* 1991). Production of endo-, exoglucanase and β-D-glucosidase by *Talaromycesemersonii* was higher on cellulose medium than on lactose and glucose containing media (Morrison *et al.,* 1987). Starch has been shown to be an effective substrate for growth and cellulolytic enzymes production by *A.phoenicis* (Sternberg *et al.,* 1977). In the order, D-glucose followed by D-saccharose, glycerol and finally D-fructose, were found to be the best carbon sources for cellulase production by *Rhodotorulaglutinis* (Oikawa *et al.,* 1998).

Among the nitrogen sources amended in the fermentation broth with 1% w/v tryptone supplementation showed maximum enzyme activities of β-glucosidases (3.13 IU/ml), xylanase 13.76 IU/ml and laccase 2.64 IU/ml. whereas the supplementation of yeast extract yielded maximum enzyme activities of exoglucanase 12.32 IU/ml, endoglucanase (12.2 IU/ml), and total protein content (13.64 mg/ml) on 6^{th} day of incubation. (Fig. 26., 27., 28., 29., 30. and 31.). The tested nitrogen sources for cellulolytic enzymes production by *A.awamori* of the current study were yeast extract, tryptone and urea at different concentrations. Among these, yeast extract and tryptone concentration was found to be optimum. The wild strain of *Chaetomiumglobosum* produced maximum yield of cellulases in the presence of peptone at 0.6% as nitrogen source followed by yeast extract, urea, KNO_3 and $(NH_4)2SO_4$ (Umikalsom, *et al.,* 1998). The increased concentration of yeast extract and peptone was inhibitory to cellulase production by *Chaetomiumglobosum* (Umikalsom *et al.,* 1998). High yields of cellulase enzyme production was found with ammonium salts by *Trichodermaharzanum* (Rousses and Raimbault 1982) and *Aspergillus fumigates* (Stewart and Parry, 1981). The addition of skim milk powder at 0.2% concentration enhanced the production of endo-glucanase and exo-glucanase by *Trichodermareesei*but it had no influence on β-D-glucosidase production (Patil *et al.,* 1995). The optimized medium composition of cellobiose-octaacetate in the presence of microcrystalline cellulose as a co-substrate, yielded several folds increased cellulolytic enzymes by *Penicilliumpurpuogenum* p-26 (Kamagata *et al.,* 1991).

Conclusion

Degradation of cellulosic materials is a multifaceted process and several microbial cellulolytic enzymes are required. The degradation of lignocellulosic materials involves a complex depolymarization process of the polysaccharide components into sugar monomers that requires several different types of enzymes. The microbial production of enzymes depends on the genetic nature of the organisms, the physico-chemical parameters, the fermentation medium components and their concentrations. Thus optimization of culture conditions is important to achieve a maximum yields for industrial applications. The main objective of this paper was to produce lignocellulolytic enzymes under optimize culture conditions by SmF using agro residual substrates like paddy straw by *Aspergillus awamori* which proved possibility of the enzyme production in economical range. Hence it can be used for large-scale production of lignocelluloses using such agro residual substrates for not only industrial purpose as forage supplement for ruminants also.

Feeding of the exogenous fibrolytic enzymes like lignocellulase were used in increased *in vitro* dry matter digestibility and *in vitro* sugar release from paddy straw.

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