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Production of Industrially Important Cellulases and Pectinases using Lignocellulosic Biomass

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ABSTRACT: Cellulases and pectinases are the two main industrially important enzymes that find significant applications in food, feed, pharmaceutical, textile and environmental sectors. In the present study, *Trichoderma asperellum* S14 was used for enzymes production in liquid state fermentation and for enzymatic hydrolysis of lignocellulosic biomass. Prior to liquid state fermentation, *Trichoderma asperellum* S14 was qualitatively screened for the pectinolytic and cellulytic activity by using plate assay method. Alkaline pretreatment (1% NaOH) was used for the pretreatment of lignocellulosic biomasses including; wheat bran, wheat straw, rice straw, rice husk and sugarcane bagasse. Maximum cellulase activity of 4.3 U/mL was observed at pH 4.8 and 30°C after 72 hours of incubation from *Trichoderma asperellum* S14 using alkali pretreated sugarcane bagasse as a substrate. While, maximum pectinase activity 9.2 U/mL obtained at pH 4.8 and 30°C after 72 hours of incubation from *Trichoderma asperellum* S14 using alkali pretreated wheat bran. Enzymatic hydrolysis was also performed using crude enzymes extract at 50°C for 72 hours. Maximum hydrolysis (70%) was observed in case of pretreated sugarcane bagasse using *Trichoderma asperellum* S14 as fungal source for enzyme production. Lignocellulosic biomasses are considered as the best feedstock used in second generation biofuel production because of their renewable resources as well as inexpensive and environment friendly nature. Present study shows the potential of *Trichoderma asperellum* S14 to degrade variety of lignocellulosic biomass efficiently and could be used to hydrolyze complex lignocellulosic materials towards the production of second generation biofuel.

Keyword: Cellulase, Pectinases, Lignocellulosic biomass, Liquid state fermentation

INTRODUCTION

Increasing fuel prices and environmental pollution is a major cause of developing alternative energy sources, especially fuel ethanol for automobiles (Sharma et al., 2022; Asgher et al., 2013; Iqbal et al., 2013). Biodegradable lignocellulosic wastes and their conversion in to biofuel at industrial level are attracting the significant interest worldwide (Moiser et al., 2005). Ethanol is a biofuel that can be produced from fermented cellulosic biomass and a great advantage of using ethanol as biofuel is that it does not contribute in global warming (Park et al., 2010). Wastes including various forests and agricultural residues are cheap, abundant and renewable resource on earth that can be used for industrial production of second generation biofuel (Awasthi et al., 2022; Shide et al., 2004). Lignocellulosic biomasses like “wheat straw, rice straw, rice husk, wheat bran and sugarcane bagasse are the by product of crop cultivation produced globally. Only a small amount of the residual plant is used as livestock feeds, farmyard manure and fuel, while a large part of the residue is discarded on the field or burned directly, which results in the waste of fuel resources as well as environmental pollution (Asgher et al., 2013).

Lignocellulosic material is the best option to use as carbon source for the microbial enzyme production and second generation biofuel production considering their availability in Asian countries, their low cost and their energy yields (Devi et al., 2022;Zhu et al., 2006). Lignocellulose is a complex matrix, that contain many different polysaccharides, phenolic polymers and proteins. Celluloses and hemicelluloses linked with lignin to form an insoluble, tridimensional complex. The complex structure is made up of 35% - 50% cellulose, 20% - 35% hemicellulose and 12% - 20% lignin (Lynd et al., 2008). Cellulose which is the key component of cell walls of land plants, is a glucan polysaccharide that contains large reservoirs of energy that provide real potential for conversion into biotechnological products. The cellulose fibers are embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin (Sharma et al., 2018; Kumar et al., 2014). Hemicellulose is a complex of polymeric carbohydrates with xylan as its major component and furthermore, a minor amount of other polymeric components: starch, pectin, proteins, minerals, ash, etc. (Badhan et al., 2007). Lignin is a heterogeneous compound which acts like a glue by filling the

spaces between and around the cellulose and hemicellulose complex. Pectin substances are the high molecular weight, negatively charged, acidic, complex glycosidic polysaccharides that are present in different percentages in vegetable and fruit plants. A pectin substance ranges from 0.5 to 4.0% of the fresh weight of plant material. The physiochemical characteristics of cellulosic biomass are responsible for the necessity for different pretreatment methods which can help in the rapid and efficient transformation of sugar polymers into fermentable carbohydrates (glucose) (Badiei et al., 2014). Prior to fermentation, pretreatment step removes lignin and hemicellulose, reduces crystallinity and increases the porosity of biomass, thus increases the accessible surface area. Hence, opting a suitable method of pretreatment plays an essential and significant in enhancing the hydrolysis efficiency and reducing the process cost as well as in reducing environmental pollution (Han et al., 2012; Badiei et al., 2014). Mostly, alkaline pretreatment is considered as more useful and effective for the subsequent degradation of agricultural wastes and herbaceous crops than that for the wood materials (Laureano et al., 2005). The pretreated lignocellulosic material is subjected to

the enzymatic hydrolysis and the resultant hydrolysates are fermented to higher value added products such as second generation biofuel using fermenting microbes (Kuhad et al., 2011).

Many microorganisms mostly fungi can produce the variety of enzymes. Among those *Trichoderma*, *Aspergillus* and *Penicillium spp.* are some of them and *Trichoderma* is one of the most efficient cellulases producer which has been extensively researched for the production of cellulose and pectin degrading enzymes in liquid or solid state fermentation (Iqbal et al., 2013). Commercial enzyme preparations are expensive, because they are produced from refined substrates and usually patented organisms. Therefore cheaper substrates for enzyme production and fungi with good enzyme-producing capacity is usually investigated. *Trichoderma* fungi are the most abundant filamentous fungi in nature. *Trichoderma* success on land is reflected both by their efficient utilization of variety of substrates as well as their secretion capacity for antibiotic metabolites and enzymes (Druzhinina et al., 2006). Cellulases and pectinases are industrially important hydrolytic extracellular enzymes which have the ability to completely degrade

lignocellulosic materials (Beguin et al., 1994). These enzymes have wide range of applications in textile, food and feed industry, paper recycling, brewing and in research and development (Imran et al., 2016). Present study reveals the ability of *Trichoderma asperellum* S14 of utilizing different types of lignocellulosic biomass which include sugarcane baggass, rice husk, rice straw, wheat bran and wheat straw and consequently produce combination of enzymes in liquid state fermentation. These enzymes can be used to produce fermentable sugars for biofuel production.

MATERIALS AND METHODS

Culture collection

The fungal strain *Trichoderma asperellum* S14 was obtained from the Biology laboratory of the Department of Biology of Lahore Garrison University Lahore, Pakistan. The strain was previously isolated, identified and characterized which was then cultivated on PDA media at 28°C and pH 5.5 for 5 days and stored at 4°C for further investigation.

Sample collection

Lignocellulosic substrates including sugarcane bagasse, wheat bran, wheat straw, rice straw and rice husk were purchased from the local harvest store of

Lahore, Pakistan. The lignocellulosic substrates were washed and sun dried at 50°C for 8-10 days. After proper grinding the substrates were stored in sterile polythene bags at 30°C to keep them moisture free.

Pretreatment of lignocellulosic biomass

Alkali pretreatment of lignocellulosic substrates was done by adding 0.1% NaOH solution in 40g of lignocellulosic biomass and incubated overnight at 26°C in a dark place. After 24 hours of incubation, pH of the residual substrate was checked and then washed the substrates with de-ionized water until its neutral pH was reached. Then substrates were dried at 50°C in an oven for 2 days (Han et al., 2012).

Qualitative screening of fungal strain

Trichoderma asperellum S14 was screened for its cellulolytic and pectinolytic activity on CMC and pectin containing media. For this, fungal strain was grown on CMC and pectin containing media at 28°C and pH 5.5 for 48 hours. Amoxicillin (100mg/l) was added to the media after sterilization to prevent bacterial contamination. To observe cellulolytic activity, colony diameter was measured and plates were flooded with 1% congo red for 15 minutes and then destained with 1M

NaCl solution for 20 minutes to visualize clearance zone around fungal colony. Pectin containing plates were flooded with 50 mM iodine solution for 20 minutes and then diameter of the clearance zones was measured. Clearance zone index was calculated according to the following formula:

Clearance zone index = Diameter of the colony / Diameter of the clearance zone

Quantitative screening

For quantitative estimation of pectinase and cellulase enzymes, enzyme assay was performed. Enzyme assay for pectinase and cellulase was performed by DNS method of Miller (1959) after every 24 hours for 5 days. For this crude filtrates were centrifuged at 12000 rpm for 15 minutes at 4°C. About 0.5% pectin and CMC substrates were separately prepared by dissolving in 0.05M citrate buffer at pH 4.8. Appropriately diluted the enzyme solution with citrate buffer and incubated in water bath at 60 minutes at 40°C. Then 3mL of DNS reagent was added to stop the reaction. Optical density was measured at 540 nm after cooling the solution. Enzyme activities were calculated according to the following formula:

Enzyme activity $Y = X + 0.092 / 0.335$

One unit (U) of enzyme activity was defined as the amount of enzyme which releases 1 μ mol of polygalacturonic acid and glucose per minute for pectinase and cellulase assay respectively.

Preparation of Spore Suspension

Trichoderma asperellum S14 was cultivated in PDA slants and spore suspension was prepared by using 0.9% saline solution of NaCl. Spore suspension was then serially diluted with more saline solution. Spore concentration of the 2 mL inoculum was determined by using hemocytometer which was 10⁻⁶ to 10⁻⁷ spores/mL.

Enzyme production under Liquid State Fermentation

About 2 mL spore suspension was taken and inoculated in to 250 mL Erlenmeyer flask containing 100 mL of freshly prepared Mandel's media. Mandel's media was made by taking 0.14 % (NH₄)₂SO₄, 0.2 % (KH₂PO₄), 0.03 % Urea, 0.2 % CaCl₂, 0.03 % MgSO₄.7H₂O, 0.1 % Peptone and 0.1 mL trace metal solution. 1% lignocellulosic biomass was added as carbon source and 2 mL Tween 80 was added in the reaction mixture. Five pretreated lignocelluloses including wheat bran, rice husk, rice straw, wheat straw and sugarcane bagasse were used as substrates in liquid state fermentation.

These media flasks were incubated for 5 days in a shaking incubator at 30°C and 150 rpm. Pectinase and cellulase activities were measured after every 24 hours.

Enzymatic Hydrolysis

For enzymatic hydrolysis of pretreated substrates *Trichoderma asperellum* S14 was grown on Mandel's media at 30°C for 72 hours in an Erlenmeyer flask. Crude enzyme filtrate was obtained and centrifuged at 14000 rpm for 10 minutes at 4°C. 10 g of alkali pretreated substrates were taken in separate flasks and 100 mL of crude enzyme hydrolyzate was added in them then incubated in shaking incubator at 250 rpm and 50°C for 72 hours. Again 2 mL of sample was taken out from the flask after each 24 hours and 50 mM citrate buffer with pH 5.5 was added in it and centrifuged at 16000 rpm for 10 minutes. The amount of reducing sugars was calculated using 3, 5 dinitrosalicylic acid (Miller, 1959).

RESULTS AND DISCUSSION

Screening of *Trichoderma asperellum* S14 for cellulase and pectinases production

Trichoderma asperellum S14 was at first qualitatively screened for their cellulase and pectinase production on agar plates using carboxymethyl cellulase and pectinases as sole carbon source. Significant growth was observed on both carboxymethyl cellulose and pectin containing media. Colony diameters were calculated after 48 hours of incubation. Clearance zones were formed on treatment with 1% congo red solution and iodine solution in case of cellulase and pectinases production respectively. These clearance zones determine the ability of cellulose and pectin hydrolysis by *Trichoderma asperellum* S14. In case of cellulose and pectin hydrolysis, zones of hydrolysis were greater than colonies diameter. Clearance zone index was calculated by dividing diameter of fungal colony by diameter of clearance zone. Fig. 1 is demonstrating the results of primary screening of *Trichoderma asperellum* S14 for cellulase and pectinase activity on cellulose and pectin agar.

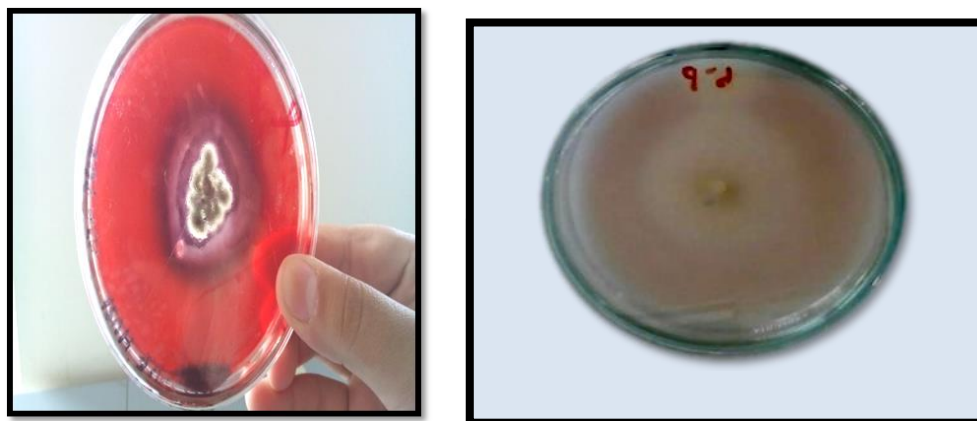


Fig. 1: Primary screening of *Trichoderma asperellum* S14 on cellulose and pectin agar.

Pretreatment of lignocellulosic biomass

It was observed that physical and chemical pretreatment of lignocellulosic biomass including rice husk (Fig. 2) resulted in removal of lignin and hemicelluloses efficiently, while at the same time pretreatment also proved helpful for the loosening in structure of lignin and decrease the integrity of cellulose that improves the porosity characteristic of substrate and thus it increased the availability of monomeric sugars. A significant increase in enzyme activity was observed when pretreated substrates were inoculated with freshly prepared inoculum of *Trichoderma asperellum* S14. From previous study, it was found that rate of enzymatic activity increases when size of the wheat straw substrate reduces straw (Han et al.,

2012). Moreover, as compared to acid or oxidative reagents, in breaking the ester bonds between lignin, hemicellulose and cellulose efficiently, and avoiding fragmentation of the hemicellulose polymers afterwards, alkali treatment is known to be the most operative method (Gaspar et al., 2007). Enzyme activity increases by increasing the time of pretreatment. Previous report shows 2.5, 1.6 and 1.7 fold increase in endoglucanase, exoglucanase and β -glucosidase activity respectively by using 0.1 M NaOH pretreated maize straw (Goyal and Soni, 2014). Present study shows that by using 0.1% (w/v) NaOH and giving 24 hours for pretreatment, highest value of cellulase activity with sugarcane bagasse which was 4.3 U/mL and 9.2 U/mL in case of pectinase activity can be achieved.

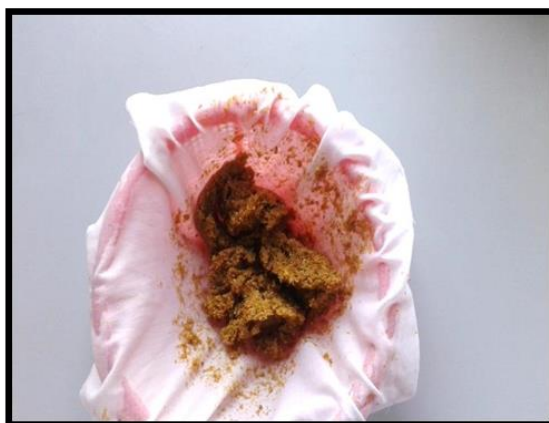


Fig. 2: Pretreated rice husk after washing with deionized water

Liquid State Fermentation

Production of two fungal enzymes namely cellulase and pectinase enzyme was determined by liquid state fermentation using 2 mL of *Trichoderma asperellum* S14 spore suspension as inoculum in 100 mL Mandel's medium.

Effect of different carbon sources and Fungi on cellulase production

Carbon source in the broth medium affects considerably in the synthesis of hydrolytic enzymes by fungi (Gorems, 2014). As different substrates, rice straw, rice husk, wheat bran, wheat straw and sugarcane bagasse were used as sole carbon source separately therefore the rate of enzyme production was different in all experiments. Pretreated lignocellulosic biomass including sugarcane bagasse, rice husk and rice straw were found to be the good

carbon sources to induce the production of cellulases. In present research, sugarcane bagasse gave the best results as maximum cellulase production 4.3 U/mL was observed in LSF using *Trichoderma asperellum* S14 at pH 4.8 and temperature 30°C after 72 hours (Fig. 3). Then rice husk, rice straw and wheat bran also produced the significant yields 4.24, 4.20 and 3.70 U/mL of cellulase respectively (Fig. 4, 5, 6). However, minimum yield 3.17 U/mL was obtained from wheat straw under same fermentation conditions (Fig. 7). Present study shows higher values of cellulase activity in all experiments as compared to previous researches for example, in a research, cellulase and FPase enzyme activities were investigated as a measure of suitable substrate pre-treatment and optimum condition for hydrolytic enzyme production.

The highest enzyme activity was found to be 1.217 U/mL for cellulase with *Trichoderma harzianum*S14 using steamed, NaOH pretreated sugarcane bagasse as substrate. However, wheat bran and wheat straw gave average of 3.70 and 3.17 U/mL of cellulase activity respectively. Sibtain studied maximum 0.28 U/mL cellulase activity by using alkali pretreated wheat brand as carbon source and *Trichoderma harzianum* as fungal source (Sibtain at al., 2009). In another study, 1.27 U/mL enzyme activity was recorded in alkali pretreated sugarcane bagasse using *Trichoderma viridei* in liquid state fermentation. While H₂SO₄ pretreated bagasse gave 0.28 U/mL enzyme yield and minimum enzyme activity was reported with H₂O₂ pretreated bagasse with *T. viridei* in

liquid state fermentation. Moreover, Gorems found maximum 0.6 U/mL enzyme activity of cellulase using pure cellulose as carbon source at 30°C temperature and pH 5.5 and *Trichoderma* fungi as fungal source (Gorems, 2014). Highest value of enzyme activity can be observed by who was able to produce maximum 3.29 U/mL of cellulase by using coir waste as a carbon source and *Aspergillus niger* as fungal source in liquid state fermentation at pH 6 after 72 hours of incubation. From previous findings it can be concluded that present study gave the highest enzyme activity values with alkali pretreated biomasses and *Trichoderma asperellum* S14 under controlled conditions.

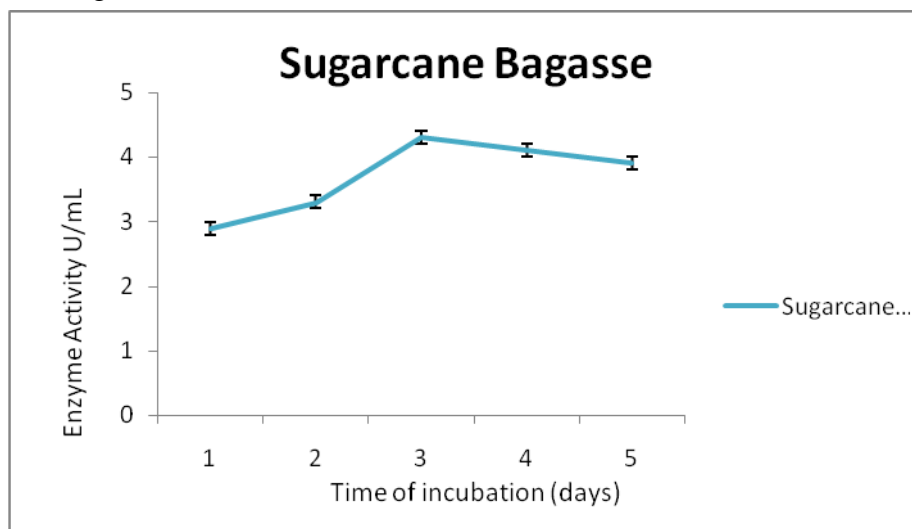


Fig. 3: Enzyme activity in U/mL of cellulase enzyme using *Trichoderma asperellum* S14 LGML-1 with sugarcane bagasse as substrate.

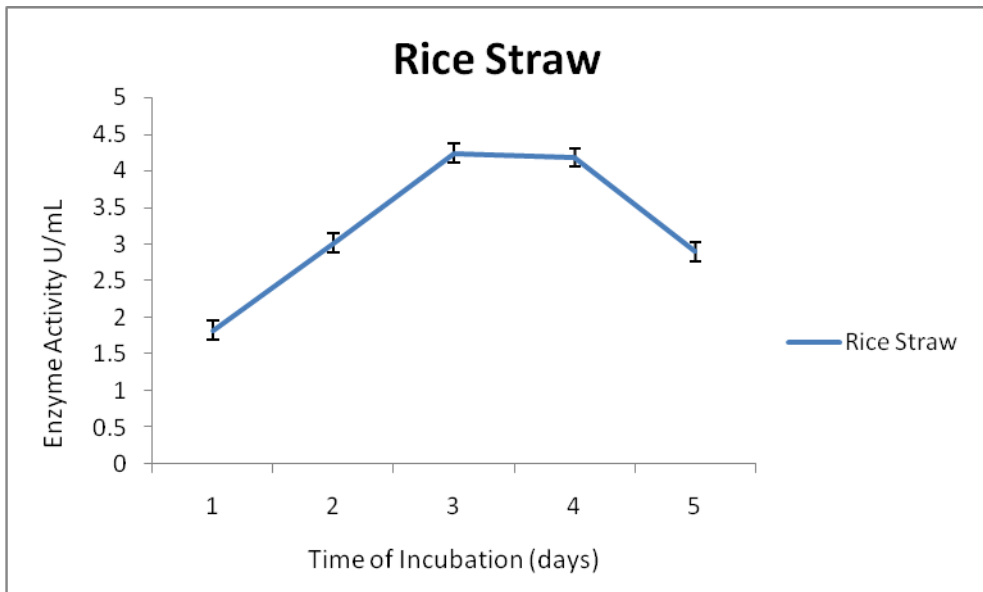


Fig. 4: Enzyme activity of cellulase by using *Trichoderma asperellum* S14 LGML-1 with rice straw as a substrate

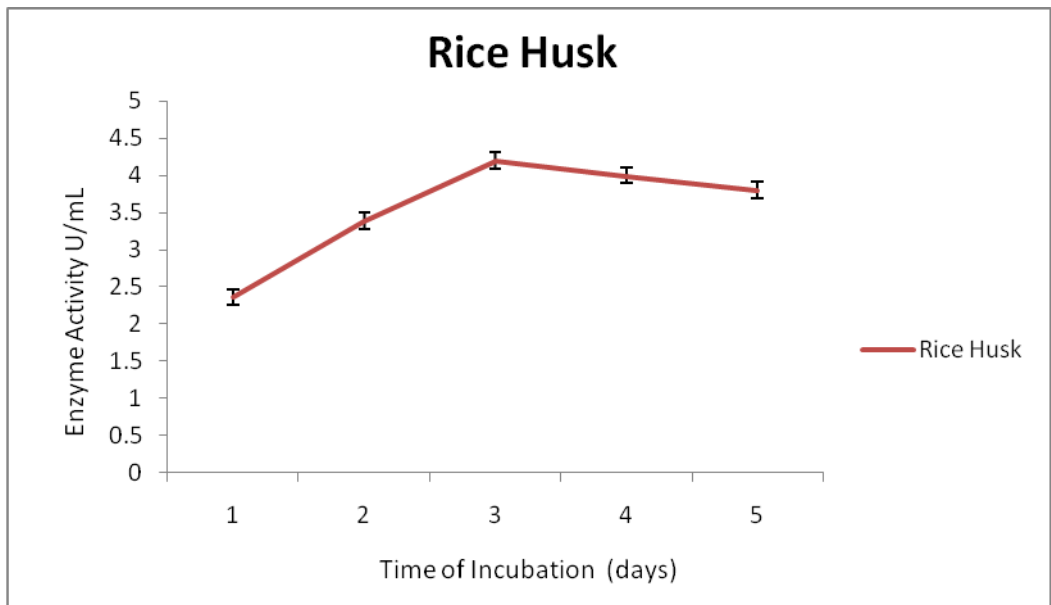


Fig. 5: Enzyme activity of cellulase using *Trichoderma asperellum* S14LGML-1 with rice husk as a substrate

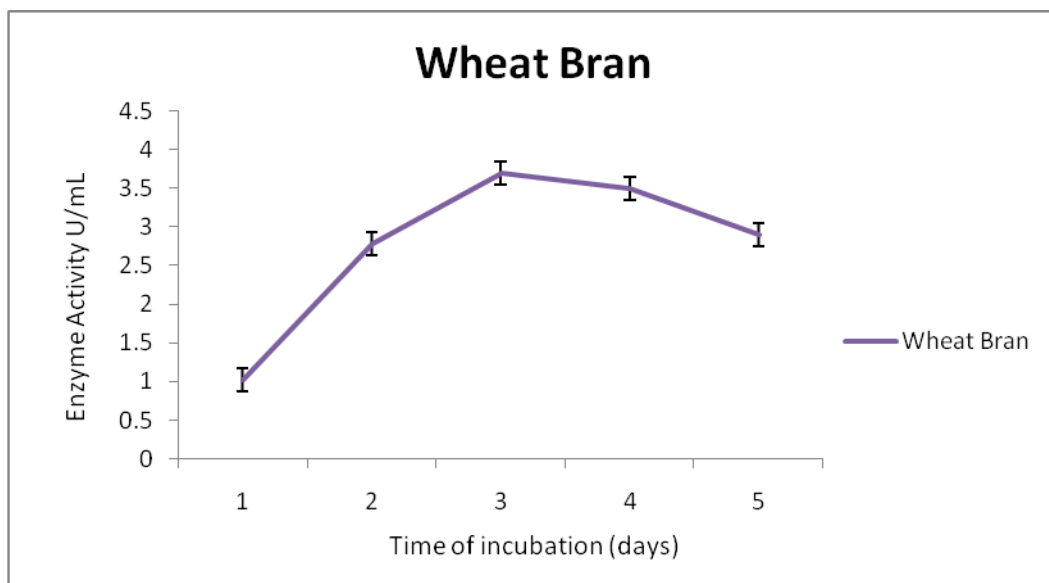


Fig. 6: Enzyme activity of cellulase using *Trichoderma asperellum* S14 LGML-1 with wheat bran as a substrate

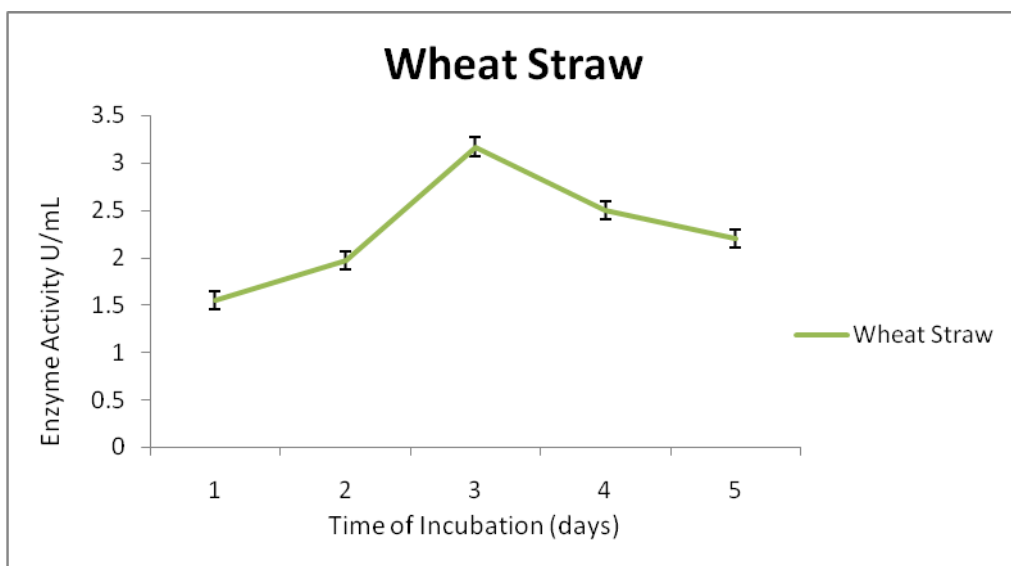


Fig. 7: Enzyme activity of cellulase by using *Trichoderma asperellum* S14LGML-1 with wheat straw as a substrate

Effect of different carbon sources and Fungi on pectinase production

On the other hand, pectinase activity was found maximum with wheat bran which was recorded as 9.2 U/mL till 72

hours of incubation at 30°C temperature and 150 rpm in a shaking incubator. Hence wheat bran is the best inducer of pectinase enzymes with *Trichoderma asperellum*. Moreover, rice husk and

rice straw were also the good inducers of pectinases in case of *Trichoderma asperellum* S14as their enzyme activities were 9.17 and 9.14 U/mL respectively in similar culture conditions. But sugarcane bagasse and wheat straw gave comparatively lesser values which were 8.9 and 9.08 U/mL respectively but these values were greater than previous researches. Such as highest pectinase activity 8.2 U/mL was recorded with agro waste using *Trichoderma viridei* in submerged fermentation (Ansari et al., 2014).

Enzymatic hydrolysis

Sugarcane bagasse has high cellulose contents 42 % (Kim and Day, 2011) as compared to wheat and rice straw therefore more enzymatic hydrolysis was recorded as compared to other cases. Such as 70% hydrolysis was observed using alkali pretreated sugarcane bagasse which was greater than rice husk, rice straw, wheat straw and wheat bran in which 66.5%, 64%, 60% and 40% hydrolysis was recorded respectively at 50°C temperature and pH 5.5 after 72 hours. Hydrolytic enzymes of *Trichoderma asperellum* S14gave the best results as compared to other researches such as highest results were observed in another investigation (Irfan et al., 2013) in which maximum hydrolysis 64%, 40% and 34% with

sugarcane bagasse, rice straw and wheat straw respectively was observed with *Saccharomyces cerevisiae*. Moreover, another study found 42.7% hydrolysis with sugarcane bagasse using *Trichoderma viridei* as fungal source while in another report maximum 35.5, 33.5 and 25.5% hydrolysis was observed with sugarcane bagasse, wheat straw and rice straw respectively (Akhtar et al., 2001).

CONCLUSION

Wheat bran and sugarcane bagasse can provide an economical and environmental advantage as carbon sources for the production of pectinase and cellulase enzymes respectively, using *Trichoderma asperellum*S14. Under optimized fermentation conditions, best enzyme activity 9.2 U/mL in case of pectinase and cellulase activity 4.3 U/mLwas recorded using *Trichoderma asperellum*S14. Then enzymatic hydrolysis of same pretreated substrates including sugarcane bagasse, rice husk, rice straw, wheat straw and wheat bran, was performed with *Trichoderma asperellum* S14and 70% hydrolysiswas obtained in case of pretreated sugarcane bagasse. Such high values under moderate conditions suggests that these microbial enzymes (cellulases and pectinases) can be used

for second generation biofuel production, in animal feed industries, agricultural biomass refining, textile industries and brewing, wine making and fruit juice clarification.

Future prospective

In the present study, results of highest enzyme activity and hydrolysis suggests a great advantage of using sugarcane bagasse, rice husk, rice straw, wheat straw and wheat bran as carbon source and *Trichoderma asperellum* S14as fungal source in enzyme production and hydrolysis of pretreated substrates which can be used for second generation biofuel production in future.

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