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Optimization of Fermentation Conditions for Enhanced β-Glucosidase Production from Cellulolytic *Aspergillus terreus* Strain PPCF using Agro-Residues

Heena Parveen, Lakshmi Tewari

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Abstract Lignocellulosic biomass represents a promising option as carbon source the cellulase enzyme production but most of the microbial cellulases have been reported with low β-glucosidase activity. Efforts were, therefore made in the present study for the selection of an efficient β-glucosidase producing fungal strain and optimization of fermentation conditions for the enzyme production using wheat bran as a sole carbon source. The Carboxy methyl cellulose hydrolyzing fungus showing maximum extracellular β-glucosidase activity (0.517 Uml^{-1}) in crude culture filtrate was selected and identified as *Aspergillus terreus* PPCF by 5.8S ITS rRNA- gene sequencing. An increase in enzyme activity from 0.517 Uml^{-1} to 1.19 Uml^{-1} was achieved in 7 dunder optimized liquid conditions (pH 7.0) using wheat bran (3%) w/v) as sole carbon source and ammonium sulfate (0.5% w/v) as nitrogen source with 8% inoculum size (CFU 3.2×10^8 ml^{-1}) at 27 ± 2°C. During solid-state fermentation with 1:7 solid to a liquid ratio the enzyme activity was further enhanced to 2.01 Uml^{-1}. Solid state fermentation utilizing agro-wastes as natural-substrate is an appropriate cost-effective technique for fungal enzyme production that directly emphasizes on the management of agricultural solid waste. The study also suggests wheat bran as an appropriate substrate for commercial enzyme production that can be used for cellulosic bioethanol production.

Keywords β -glucosidase, *Aspergillus terreus* PPCF, LSF, SSF, Lignocellulosic biomass.

Heena Parveen*, Lakshmi Tewari
Department of Microbiology, College of Basic Science and Humanities, G. B. Pant University of Agriculture and Technology, Pantnagar 263145, Uttarakhand, India
e-mail: heenahussain0000@gmail.com
*Corresponding author

Introduction

Energy consumption particularly in the form of petroleum based fuels in transportation, industries and in many other activities has been an established practice since many years. But, this conventional approach is becoming a notable global threat as it releases greenhouse gases (GHG) into the atmosphere posing environmental and health hazards (Iwo et al. 2016). These consequences have led to discover alternative means for achieving sustainability in the energy production and also in energy consumption. In this regard, there are many alternatives ; one among them is energy production from plant biomass (lignocellulose) which is the most abundant source in nature (Shen et al. 2017). There are various forms of biomass resources in the world which act as an alternative source for bioethanol production include agriculture (crop based) residues, municipal solid waste and woody plant residual. These biomass resources seem to be the largest and most promising future resources for biofuels production. As the world is marching towards achieving carbon neutrality, it is very essential to focus on the conversion of residual plant waste into more useful clean energy (Lynd et al. 2002, Sun and Cheng 2002). Large quantities of agricultural by-products (e.g. rice and wheat straws) are

produced every year and most of it is left on the field or burned. The remains of plants left over, being processed by detritivores and saprophytes in detrital food web by using their metabolites including hydrolytic enzymes (Himmel et al. 2010). Utilization of microbial enzymatic action to degrade inexhaustible cellulosic biomass for the production of industrial chemicals and preparation of cellulose polymers will help to meet energy and food demands (Ja'afarul and Fagade 2010). The enzyme catalyzed hydrolysis of cellulose and hemicellulose to fermentable sugars is accomplished in nature via the collective action of multiple carbohydrate-active enzymes, typically acting together as a cocktail with complementary, synergistic activities (Lynd et al. 2002, De Souza 2013, Gao et al. 2013).

Several cellulolytic filamentous fungi including members of Ascomycetes and Basidiomycetes have been reported to produce cellulase enzyme with the ability to penetrate cellulosic substrates through hyphal extensions, thus often presenting their cellulase systems in confined cavities within cellulosic particles (Gautam et al. 2011). Microbial cultures need optimum culture conditions for their growth and cellulase enzyme production which vary among different isolates. The major components of the enzyme production medium such as carbon, nitrogen and phosphorus sources and physical parameters like temperature, pH and incubation period were found to be critical in affecting the microbial cellulase production and hence there is a need to optimize these fermentation conditions for enzyme production by each newly isolated cellulolytic strain (Nathan et al. 2014).

Most cellulases consist of a general feature and possess a modular structure which includes both catalytic and carbohydrate-binding modules (CBMs). The CBM help in binding of the enzyme to the cellulosic surface and facilitate cellulose hydrolysis by bringing the catalytic domain in close proximity to the substrate (insoluble cellulose). The presence of CBMs is particularly important for the initiation and action of exoglucanases (Teeri et al. 1998). β-glucosidases are crucial for effective hydrolysis as they alleviate the inhibition of the cellobiohydrolases and endoglucanases by reducing cellobiose accumulation and thereby, avoiding decreased hydrolysis rates of cellulose over time. However, β-glucosidases are often themselves inhibited by their product glucose making β-glucosidase the rate-limiting enzyme. Maintaining a high hydrolysis rate of cellulose ultimately requires highly efficient β-glucosidases that can tolerate glucose at high levels.

The production of enzyme is not only influenced by the nutritional and physiological parameters used during growth and fermentation, but is an innate genetic characteristic of every individual system. Moreover, cellulase expression can be further regulated under optimized conditions achieving sufficiently high enzyme yield. Diverse natural substrates including various agro-wastes are widely used for production of various fungal metabolites/enzymes which leads to their utilization for valuable product formation and creates a strategy for solid waste management. Considering the need for identification of an efficient β-glucosidase producing strain, newly isolated cellulolytic fungal and bacterial cultures were screened. The main emphasis of this research is to increase the production of β-glucosidase enzyme because it is the vital component in cellulolytic mechanism (saccahrification process) and therefore considered to be the key enzyme in determining the cellulase efficiency and the bottle neck in bioethanol production through biomass conversions (Ahmed et al. 2017).

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Materials and Methods

Isolation and selection of cellulolytic cultures

Thirty five indigenous microbial cultures (bacteria and fungi) were isolated from diverse natural sources viz. ground soil, cow dung and paper wastes by serial dilution pour plate technique using Carboxy methyl cellulose (CMC) agar medium containing (%, w/v) CMC, 1.0 ; peptone, 1.0 ; K_2HPO_4, 0.2 ; $(NH_4)_2SO_4$, 0.25 ; $MgSO_4.7H_2O$, 0.03 ; and agar, 2.0, pH 7.0. For isolation of bacterial cultures, the inoculated plates

were incubated for 48 h while in case of fungi the plates were incubated for five day at 27 ± 2°C till the appearance of sufficient growth. The purified colonies were screened for their cellulase activities by flooding with 0.1% congo-red solution for 5 min followed by de-staining with 1N NaOH solution for 15–20 minutes. The qualitative measure of extracellular cellulase activity is the presence of clear yellow zone around the growing colony against the dark red background indicating the CMC hydrolyzing capability of the culture. On the basis of hydrolysis zone formation during the plate assay (qualitative testing), the index of relative enzyme activity (1_{CMC}) was calculated.

Production of crude enzyme

Out of thirty five indigenous isolates, only twenty two cultures showing clear zone formation on CMC agar plates during qualitative assay were selected for quantification of the enzyme. Overnight grown active bacterial culture (OD=0.6) was used as inoculum for bacterial cellulase production while aqueous spore suspension (CFU=3.2×10^8 ml^{-1}) was used as fungal inoculum for enzyme production. The active bacterial cultures were inoculated and grown in enzyme production medium (CMC broth) at 120 rpm in incubator shaker at 27 ± 2°C for 24 h ; while fungal cultures inoculated in the same enzyme production medium were incubated under static conditions for 168 h. After incubation, the cultures were centrifuged at 8000 rpm (4°C) for 12 min. The supernatant was collected and used as crude enzyme for estimation of overall cellulase (FPase) and β-glucosidase enzyme activities for selection of an efficient cellulolytic microbial culture.

Estimation of enzyme (FPase and β-glucosidase) activities

Overall cellulase (FPase) and β-glucosidase activities in crude enzyme were analyzed following the methods as described earlier (Ghose 1987, Wood and Bhat 1988). Reaction mixture for estimating FPase activity contained 0.5 ml of appropriately diluted crude enzyme in 1.0 ml of 0.05 M sodium acetate buffer (pH 5.5) with 50 mg of Whatman No. 1 filter paper strip ; 1.0 × 6.0 cm and then incubated at 50°C for 60 min. After incubation, 3 ml DNS reagent was added in the reaction tube and boiled for 5 min to measure the reducing sugars liberated during the reaction by the development of color. The boiled tubes were cooled immediately and absorbance was recorded at 540 nm. The amount of glucose liberated were quantified using standard curve of glucose. For β-glucosidase assay, the diluted crude enzyme (0.5 ml) was incubated with 0.5 ml of pNPG substrate dissolved in 0.05 M acetate buffer at 50°C for 30 min. After incubation chilled 1M sodium carbonate solution was added to stop the reaction and absorbance was recorded at 405 nm. In this case, the values were quantified using para-nitro phenol (pNP) standard curve.

Characterization and identification of potential β-glucosidase producing strain

The filamentous cellulolytic fungal isolate PPCF showing maximum β-glucosidase activity in crude culture filtrate was characterized using morphological, biochemical and molecular markers. Molecular characterization was done by 5.8S ITS rRNA gene sequence analysis. Genomic DNA was extracted, purified and PCR amplification was done by using ITS forward primer, FP (5'–GRAAGNAHADGTVGKAAYAWSG–3') and ITS reverse primer, RP (5'–TCCTNCGYTKATKGVTADGH–3') with total reaction volume of 100 µl containing DNA (1 µl), FP (400 ng), RP (400 ng), dNTPs (10 mM each) 4 µl, 10X ChromTaq DNA Polymerase Assay Buffer (10 µl), ChromTaq DNA Polymerase Enzyme (3U/µl) 1µl and water to make up volume (100 µl). After preparation of reaction mixture, PCR profile was run with total 35 cycles containing denaturation at 95°C for 5 min, annealing for 30 sec and extension for 7 min at 72°C. PCR amplified products were then purified, sequenced and compared with the sequences in nucleotide data base (NCBI) using the BLAST algorithm. Multiple sequence alignment was carried out with CLUSTAL W. Identification of the culture was done by neighbor joining phylogenetic analysis. Bootstrap analysis with 1000 replicates was performed to assess the support of the clusters. MEGA program was used for phylogenetic analysis (Tamura et al. 2013).

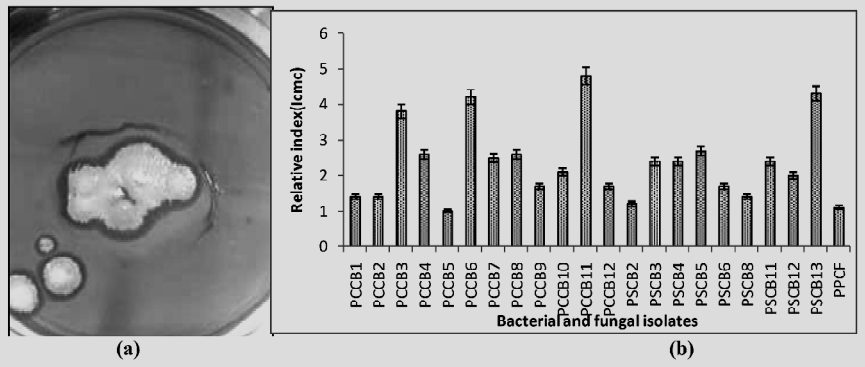

Fig. 1. Zone of hydrolysis showing extracellular cellulase activity on CMC agar plate (a), relative cellulase enzyme activity indices (I_{cMC}) of selected native cellulase producing cultures (b).

Optimization of culture conditions for β-glucosidase production

Liquid state fermentation (*LSF*)

Enzyme production conditions were optimized using one variable at a time to raise the enzyme productivity and yield from the selected fungal isolate PPCF via optimizing various parameters in liquid production medium such as size of the culture inoculum (spore suspension containing 3.2×10^8 spores ml^{-1}) varying from 2–10% v/v, each type of carbon source (wheat bran, rice bran, rice husk, wheat straw, banana pulp, and cellulose, (1% w/v), concentration of the optimized carbon source varying from 0.5 to 3.0% (w/v), type of nitrogen source (urea, peptone, ammonium sulfate and ammonium nitrate, at the rate of 0.25 %, w/v) and concentration of the selected nitrogen source varying from 0.125 to 1.5 % (w/v), pH of the medium ranging from 4.0 to 10.0 and incubation temperature ranging from 27 to 50°C. Initial pH of the culture medium was adjusted by using acetate buffer (pH 4, 5), phosphate buffer (pH 6, 7, 8), tris (hydroxymethyl) amino methane (pH 9) and alkaline borate buffer (pH 10). The inoculated flasks were incubated for 168 h at 27 ± 2°C and β-glucosidase activity was assayed as described earlier.

Solid state fermentation (*SSF*)

The lignocellulosic solid substrate (wheat bran) selected as optimum carbon substrate for β-glucosidase production during liquid state fermentation was further evaluated for enzyme production through solid state fermentation under the optimized culture conditions as obtained during LSF keeping wheat bran (substrate) concentration at a constant rate of 3% w/v, the moisture content in the medium was varied. Low level of liquid was kept during enzyme production so that organism flourishes over solid substrate and maximum yield was obtained. Different solid to liquid ratio (1:5, 1:7, 1:9, 1:11 and 1:13) conditions were provided to escalate the enzyme production. The solid state cultures were incubated at 27±2°C for 7 d. Differences in growth rate on solid substrate amended with different water levels ; enzyme was extracted from all incubated flasks and maximum activity and productivity was assayed.

Statistical analysis

Analysis of variance (ANOVA) was done with statistical software SPSS Statistics (version 19.0). All the experiments were conducted in triplicates and the data are shown as mean values±standard deviation (SD). Results are considered statistically significant at the 95% confidence interval ($p < 0.05$).

Results

Selection of cellulase producing cultures

Thirty five fungal and bacterial strains isolated from diverse source (soil, cow dung and paper waste) on agar plates were screened qualitatively for hydrolyz-

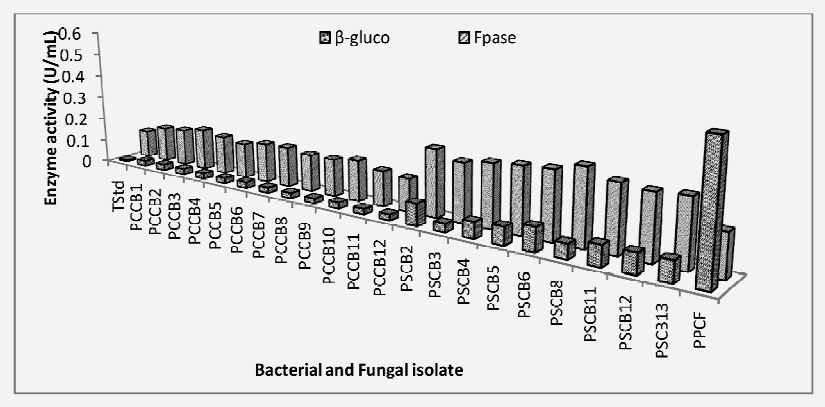

Fig. 2. Extracellular cellulase (FPase and β-glucosidase) activities in crude culture filtrate of selected cellulolytic bacterial and fungal isolates.

ing and utilizing CMC as their sole carbon source. Differences in relative cellulase enzyme activity indices, I_{CMC} (diameter of clearance zone / colony diameter) for various isolates revealed their variable abilities to degrade CMC. Twenty two isolates showing zone of clearance more than > 1.0 cm were considered as efficient extracellular cellulase producing isolates (Fig. 1 a, b).

Identification and characterization of potential strain

All the twenty two isolates were assayed for overall extracellular cellulase (FPase), and β-glucosidase enzyme production in the CMC broth. Among all the cultures, although, the maximum FPase activity (0.277 Uml^{-1}) was shown by the bacterial isolate PSCB2 but, the fungal isolate, PPCF exhibited maximum β-glucosidase activity (0.517 Uml^{-1}) in the culture broth on the 7thd of incubation (Fig. 2). Recently, highest β-glucosidase activity (0.30 Uml^{-1}) for a bacterial culture, *Lactobacillus* LSP-24 has been reported by Gauripura and Kaliwal (2017). Another report by Lan et al. (2013) also indicated only 0.26 Uml^{-1} β-glucosidase activities from *Trichoderma viridae* culture. Thus based on quantitative measure, the fungal isolate

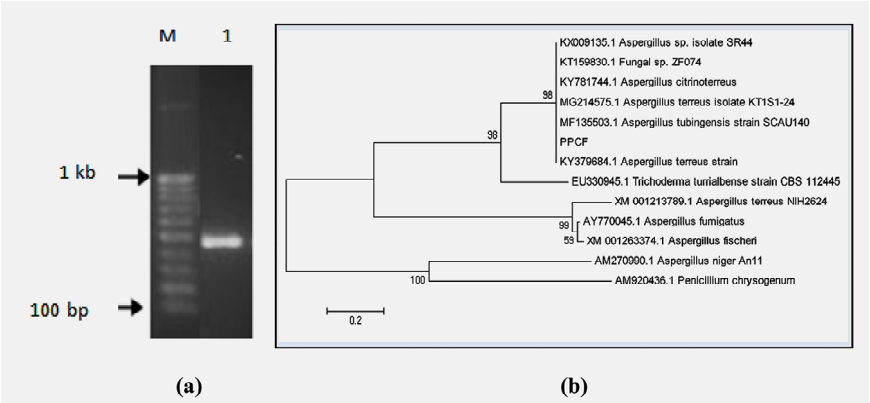

Fig. 3. PCR amplification of ITS region from fungal sample. Lane M molecular marker and Lane 1 PPCF sample. The size of PCR amplified product is ~500bp (a). Phylogenetic tree of *Aspergillus terreus* PPCF showing genetic relatedness with other fungal strains based on 5.8S ITS rRNA gene sequence retrieved from data base constructed by Neighbor-joining method (b). The bootstrap values were generated from 1000 replicates.

Table 1. Effect of *inoculum size on production of extracellular beta-glucosidase enzyme from *Aspergillus terreus* PPCF. *Cellulase production medium was inoculated with different concentration of aqueous fungal spore suspension (CFU count 3.2 ×10^8 ml^{-1}) and incubated at 27 ± 1°C for 168 h. Values are means ± standard deviation. Data with the same superscripts letter were not significantly different ($p<0.05$; Duncan method).

Inoculum size (%, V/v)	Enzyme activity (Uml^{-1})	Productivity ($UL^{-1}h^{-1}$)	Yield (Ug^{-1})
2	0.21 ± 0.05[a]	1.25 ± 0.06[a]	21.07 ±1.8[a]
4	0.31 ± 0.03[a]	1.79 ± 0.05[b]	30.66 ± 4.6[a]
6	0.46 ± 0.03[b]	2.74 ± 0.03[c]	46.39 ± 2.0[b]
8	0.60 ± 0.05[c]	3.57 ± 0.03[c]	60.08 ± 4.5[c]
10	0.53 ± 0.00[bc]	3.15 ± 0.01[d]	53.89 ± 3.17[bc]

PPCF was identified and characterized as a potential culture using polyphasic approach and selected for further studies. Molecular characterization was done based on 5.8S ITS rRNA gene sequencing. The PCR amplicons from the isolate showed the molecular weight-500 bp (Fig. 3). The phylogenetic analysis, which was based on a CLUSTAL W search, revealed that the isolate PPCF formed a clade with *Aspergillus terreus* strain KY379684.1 showing 99% sequence similarity (Fig. 3). The fungal isolate PPCF was thus identified as *Aspergillus terreus*.

Optimization of β-glucosidase production under LSF

Inoculum size

The size of inoculum is a significant factor in production of microbial metabolites. For enhancing the enzyme β-glucosidase production, the fungal isolate *A. terreus* PPCF, inoculated in the production medium (broth) with different quantity of the spore suspension (3.2 × 10^8 ml^{-1}) with rate of 2 to 10% v/v. The maximum β-glucosidase activity (0.60 Uml^{-1}) was observed at the rate of 8% inoculum in the production medium (Table 1). Khalili et al. (2017) have reported maximum β-glucosidase production by *Trichoderma harzianum* at 5% v/v concentration of inoculum loading but a decline in activity on further increase in inoculum size was observed.

Carbon source and its concentration

For optimization of carbon sources, *Aspergillus terreus* PPCF isolate was grown in production medium containing various carbon sources. All carbon sources used in this study were agro based residues. The production medium was supplemented with 1% of different carbon sources, i.e., wheat bran, rice bran, rice husk, wheat straw, banana pulp and cellulose. In wheat bran supplemented medium, the activity was reached upto 0.604 U/ml with 3.60 $UL^{-1}h^{-1}$ productivity and 60.04 Ug^{-1} yields (Fig. 4). The minimum activity was observed in rice husk inoculated medium

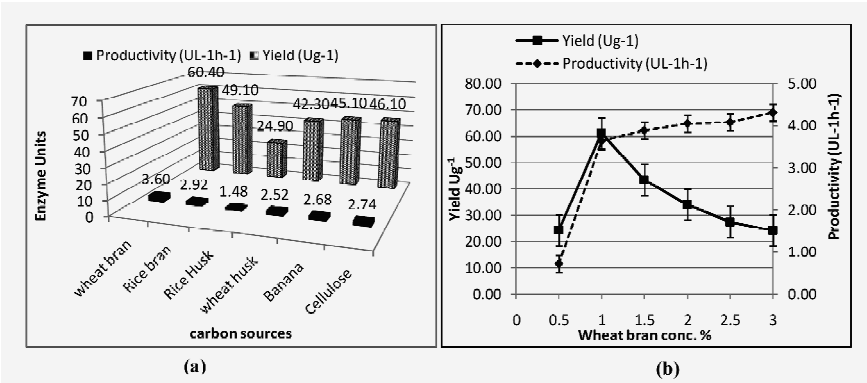

Fig. 4. Effect of different lignocellulosic substrates as carbon source (a) and concentration of selected carbon source (wheat bran) on beta-glucosidase production (b) by *A. terreus* PPCF.

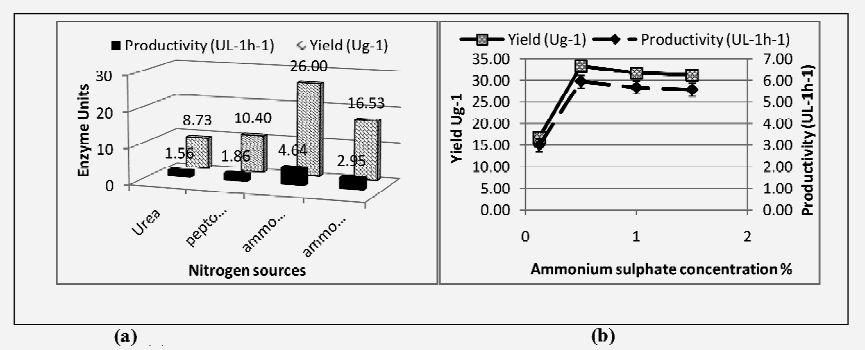

Fig. 5. Effect of different nitrogen sources (a) and concentration of selected nitrogen source (ammonium sulfate) on beta-glucosidase production (b) by *A. terreus* PPCF.

(0.249 Uml^{-1}) which leads to low productivity and yield. As wheat bran is found as best carbon source for enzyme production, it was further selected with different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%). And it was clearly depicted from (Fig. 4) that at higher concentration the enzyme activity was increased from 0.604 to 0.724 Uml^{-1} with 72.40 Ug^{-1} yield. On the contrary, Kumar and Parikh (2015), also reported the high β-glucosidase activity (75.18 ± 1.4 Ug^{-1}) in of sugarcane baggase and rice straw for inducing the enzyme production. Sørensen et al. (2014) also showed β-glucosidase production with wheat bran as carbon source by *Aspergillus saccharolyticus*. The results undoubtedly indicated the inducible nature of enzyme in presence of more substrate availability.

Nitrogen source and its concentration

Apart from the carbon source, nitrogen source is also an important factor for microbial growth and metabolite production as it the constituent of amino acids, nucleic acids, nucleotides and coenzymes of the living cell. It is also involved in governing the productivity of the method and also showed similar inducible effect as same by carbon source (Saini et al. 2017). After carbon source optimization, suitable nitrogen source for β-glucosidase production was added to the medium with inorganic (ammonium sulfate and ammonium nitrate) and organic (urea and peptone) nitrogen sources. From results, ammonium sulfate was found as the ideal nitrogen source from other sources. The enzyme activity was increased upto 0.784 Uml^{-1} in case of ammonium sulfate and decreased activity was recorded with urea as nitrogen source (0.262 Uml^{-1}) (Fig. 5). Similar findings were described by Sasi et al. (2012) which showed the highest production of cellulase enzyme by *Aspergillus flavus* utilizing ammonium sulfate as nitrogen source than yeast

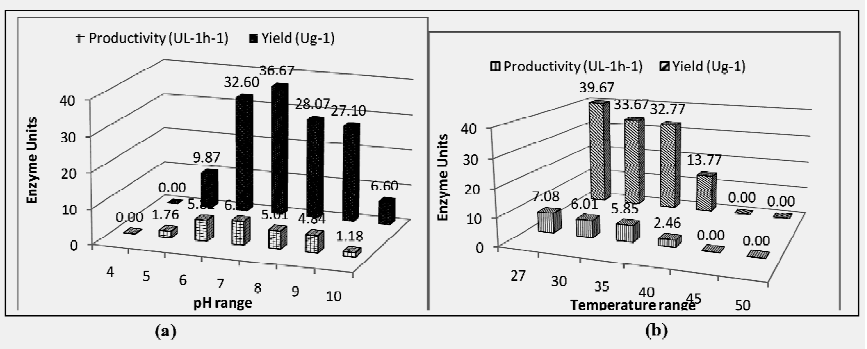

Fig. 6. Effect of pH (a) and temperature (b) on productivity and yield of beta-glucosidase by the fungal isolate *A. terreus* PPCF using wheat bran as sole carbon source during LSF.

Table 2. Effect of solid to liquid ratio on the productivity and yield of beta β-glucosidase enzyme from *A. terreus* PPCF* under solid state fermentation. *The culture was grown at 27 ± 1°C for 168 h in cellulase production medium containing different liquid amount in ml with solid residue as constant rate (3 g).

Solid: Liquid (w/v)	Enzyme activity (Uml^{-1})	Productivity ($UL^{-1}h^{-1}$)	Yield (Ug^{-1})
1:5	0.66 ± 0.2[b]	3.94 ± 0.4[a]	22.07 ±0.2[a]
1:7	2.01 ± 0.1[d]	11.94 ± 0.1[c]	66.87 ± 0.3[c]
1:9	1.45 ± 0.03[c]	8.64 ± 0.1[b]	48.40 ±0.3[b]
1:11	0.44 ± 0.09[a]	2.64 ± 0.1[a]	14.80 ±0.1[a]
1:13	0.34 ± 0.1[a]	2.01 ± 0.3[a]	11.27 ± 0.2[a]

extract. Another report also favor ammonium sulfate as nitrogen source by *T. harzianum* MTCC8230 (Kocher et al. 2008). The ammonium salts have been found as perfect nitrogen source for cellulase production by *Trichoderma* and other fungi, such as *Apsergillus fumigatus* and *Aspergillus terreus* (Stewart and Parry 1981, Mukhopadhyaya and Nandi 1999, Vyas et al. 2005).

pH and incubation temperature of enzyme production medium

Usually, each enzyme having their own optimum pH values at which their activity is highest, above and below from optimal pH the activity decreases (Lehninger et al. 1993). β-glucosidase production by isolate PPCF in liquid state fermentation was examined at various buffered pH values ranging from 4.0 to 10.0. The maximum β-glucosidase activity (1.10 Uml^{-1}) was observed at pH 7 and still production was observed upto pH 10.0 (0.198 Uml^{-1}) (Fig. 6). This specifies that the fungal isolate need neutral conditions for maximum enzyme production but also able to tolerate alkalinity of the medium. This value is in accordance with Akiba et al. (1995) who reported that the optimum pH for cellulase production by *A. niger* was between 6.0 and 7.0. Similar results were showed by El-Hadi et al. (2014) which indicated maximum cellulase production by *Aspergillus* at pH 7.0. Also, Bansal et al. (2012) found the highest cellulases production by *A. niger* NS-2 was at pH 7.0. For microorganisms, incubation temperature is also a crucial factor in the metabolic activities and minute changes in temperature can affect metabolic production. Each species prefer varied range of temperature for β-glucosidase production and usually this temperature is related to the optimum temperature for microorganism growth. Presently, maximal production of β-glucosidase was found at 27°C with increase in enzyme activity up to 1.19 Uml^{-1} and 39.67 Ug^{-1} yield. The fungal isolate PPCF showed production up to 40°C with very less activity (0.413 Uml^{-1}) (Fig. 6). This indicate that the fungal isolate require moderate range of temperature for growth and metabolic process. At higher temperature, no enzyme was produced with no growth rate of fungal isolate PPCF which revealed mesophilic nature of isolate PPCF.

β-glucosidase production under SSF

In present investigation, solid was kept at constant rate by changing liquid content amount. Maximum activity was achieved at low moisture content (65%) i.e. ; at 1:7 ratio (2.01 Uml^{-1}) and yield was increased upto 66.87 Ugm^{-1}. As, the moisture content increases, the activity start decreasing and the fungal growth was also slowdown (Table 2). This clearly depict that fungal system is suited for solid state fermentation technique for extracellular enzyme production which allowed fungal growth by providing direct hyphal contact with wheat bran. Solid state fermentation process is done on the solid substrates under water limited condition i.e., water is available in the bound form only, which acts as both source of nutrient and solid support. Dutta et al. (2008), investigated cellulase production from *P. citrinum* using wheat and rice bran and rice straw as solid substrate as these substrates supported the production of cellulases.

Discussion

Present investigation is focused on increasing β-glucosidase production using lignocellulosic biomass in a cost effective manner. The enzyme β-glucosidase is the rate limiting factor in saccahrification process

as it is under feedback inhibition by its own product (glucose) thereby inhibiting the whole process. The study performs optimization of various fermentation conditions to enhance the production rate of β-glucosidase enzyme for further efficient hydrolysis mechanism. The qualitative testing of the isolates based on relative enzyme activity indices gives a preliminary measure for selection of cellulase producing micro-organisms but it may not exactly show the real cellulase activity (Potprommanee et al. 2017). Therefore, quantification of the enzyme was done for final selection of the potential cellulase producing culture and the results indicate the fungal isolate PPCF having high potential of β-glucosidase production among all isolates. Therefore, the isolate PPCF was selected for identification based on 5.8S ITS rRNA sequencing which revealed it as *Aspergillus terreus* strain PPCF. Fungal system has been more explored in high production of extra-cellular cellulase enzymes as compared to the bacterial system. For industrial cellulase, *Trichoderma reesei* is a major source but it is deficit in adequate amount of β-glucosidase activity for effective saccahrification process therefore additional β-glucosidase is required for carrying out efficient biomass hydrolysis. The fungal species *Aspergillus niger* is the major source of commercial β-glucosidase under the name of Novazym 188, where chiefly the ascomycete genus *Aspergillus* is extensively studied for β-glucosidase production (Sørensen et al. 2013). In the present study the selected native culture of *Aspergillus terreus* PPCF showed vary high initial β-glucosidase activity and therefore it was selected for enzyme production by optimizing different production parameters. The results of the present study showed that 8% inoculum size was found as optimized amount for high β-glucosidase activity. In contrast, low inoculum density was responsible for less productivity as the amount of culture is directly related to the product formation and an appropriate inoculum density is required. Fungal culture generally requires longer lag phase when transferred in fresh culture medium owing to their slower growth rate, therefore, with optimum cell density, a cumulative effect might be responsible for efficient metabolite (enzyme) production. However, when the size of inoculum was raised beyond the optimum level, the enzyme activity was decreased because of disparity between increasing concentration of inoculum and nutrient's accessibility (El-Hadi et al. 2014). Mostly, cellulases are inducible enzymes, and their production can be enhanced in the presence of various carbon sources. Various studies have been described the utilization of lignocellulosic based substrate in cellulase production. In general, wheat bran has been found to induce the production of a large variety of hydrolases in *Aspergillus* spp., including β-glucosidase. Besides carbon source, nitrogen content is also required for growth and metabolite production by organism. In this study, 0.5% of ammonium sulfate was found as effective amount in increasing yield up to 33.33 Ug^{-1} as compare to control (26.00 Ug^{-1}). The inducing effect of ammonium salts lead to the direct entry of ammonium in protein synthesis. Temperature and pH, both are also important factor for microbial growth which clearly depicts their nature for requiring mesophilic conditions or extreme conditions that even describe the nature of the metabolite. Mostly, fungus requires normal temperature and acidic / neutral conditions for enzyme production. The isolate *Aspergillus terreus* PPCF required mesophilic (pH 7 at 27°C) conditions for β-glucosidase production but in this research we found that the selected fungal isolate PPCF also able to produce enzyme in alkaline conditions also but with very low activity. This feature found unique in fungal system as most are reported for acidic conditions. For instance, β-glucosidase has been produced from *Monascus purpureus* at 30°C, *Penicillium italicum* at 28°C, *Chaetomium thermophilum* var. *copro philum* at 45°C, *Penicillium simplicissium* H-11 at 30°C, *Daldiniaes chscholzii* at 25°C, *Thermoascusaur antiacus* at 50°C, *Aspergillus oryzae* at 28°C and *Aspergillus* strain SA 58 at 35°C, although the organism grows optimally at 30°C (Daroit et al. 2008, Jeya et al. 2010). A higher temperature is expected to denature enzymes due to heating effects (Yoon et al. 2014). Usually microbes are not able to produce high amount of β-glucosidase e.g., cellulase hyper-producer species, *Trichoderma reesei*, lacks sufficient β-glucosidase activity (Stutzenberger 1990). Thus, searching a high β-glucosidase producing microorganism is a matter of concern for researchers. Several microbes have been producing β-glucosidase including fungi, yeast and bacteria by utilizing solid state fermentation (SSF) or submerged fermentation (SMF) methodology (Pandey et al. 1999, Raza et al. 2011). Generally SMF fermentation

technique is best suited for bacteria and other microbes that need high moisture content for production of enzymes, bio-actives and metabolic wastes. But the disadvantage is that the production rate is low and foaming issue which depletes the dissolved oxygen in the medium (Singhania et al. 2013). In SSF, solid substrate is required for the growth of microorganism such as cassava cake, wheat bran, rice straw, castor bean cake, sugarcane bagasse, or corn husk solely or in combination. SSF is more suited for cultivation of microorganisms with less moisture content requirement such as fungi. The advantages of SSF are high productivity, cheap substrate utilization, low energy requirement, minimal water output and lacking of foam up (Brijwani and Vadlani 2011). The fungal β-glucosidase production in liquid medium was increased under optimized parameters in production medium. Enzyme production was greatly influenced by the moisture content and with low moisture content (1:7, solid / liquid ratio) during solid state fermentation, better enzyme yield was achieved as the fungus gets direct contact with the substrate (wheat bran) that helps in producing more enzymes. This study focuses on management of agro-wastes and development of a cost effective bio-process for enzyme production. The native *Aspergillus terreus* PPCF showed higher β-glucosidase activity as compared to that reported by earlier workers.

Conclusion

Present investigation is focused on increasing β-glucosidase production using lignocellulosic biomass in a cost effective manner. The enzyme β-glucosidase is the rate limiting factor in saccahrification process as it is under feedback inhibition by its own product (glucose) thereby inhibiting the whole process. The fungal β-glucosidase production in liquid medium was increased under optimized parameters in production medium. Enzyme production was greatly influenced by the moisture content and with low moisture content (1 : 7, solid / liquid ratio) during solid state fermentation, better enzyme yield was achieved as the fungus gets direct contact with the substrate (wheat bran) that helps in producing more enzymes. This study focuses on management of agro-wastes and development of a cost effective bio-process for enzyme production. The native *Aspergillus terreus* PPCF showed higher β-glucosidase activity as compared to that reported by earlier workers.

References

Ahmed A, Nasim FH, Batool K, Bibi A (2017) Microbial β-glucosidase: Sources, production and applications. J Appl Environ Microbiol 5 : 31—46.

Akiba S, Kimura Y, Yamamoto K, Kumagai H (1995) Purification and characterization of a protease-resistant cellulase from *Aspergillus niger*. J Ferment Bioeng 79 : 125—130.

Bansal N, Tewari R, Soni R, Soni SK (2012) Production of cellulases from *Aspergillus niger* NS-2 in solid state fermentation on agricultural and kitchen waste residues. Waste Manag 32 : 1341—1346.

Brijwani K, Vadlani PV (2011) Cellulolytic enzymes production via solid-state fermentation: Effect of pretreatment methods on physico-chemical characteristics of substrate. Enzyme Res.

Daroit DJ, Simonetti A, Hertz PF, Brandelli A (2008) Purification and characterization of an extracellular beta-glucosidase from *Monascus purpureus*. J Microbiol Biotechnol 18 : 933—941.

De Souza WR (2013) Microbial degradation of lignocellulosic biomass. In sustainable degradation of lignocellulosic biomass–techniques, applications and commercialization. Brasil : Intech, pp 208—247.

Dutta T, Sahoo R, Sengupta R, Ray SS (2008) Novel cellulases from an extremophilic filamentous fungi *Penicillium citrinum* : Production and characterization. JIMB 35 : 275—282.

El-Hadi AA, El-Nour SA, Hammad A, Kamel Z (2014) Optimization of cultural and nutritional conditions for carboxy methyl cellulase production by *Aspergillus hortai*. J Radiat Res Appl Sci 7 : 23—28.

Gao L, Gao F, Zhang D, Zhang C (2013) Purification and characterization of a new β-glucosidase from *Penicillium piceum* and its application in enzymatic degradation of delignified corn stover. Bioresour Technol 147 : 658—661.

Gautam SP, Bundela PS, Pandey AK, Khan J (2011) Optimization for the production of cellulase enzyme from municipal solid waste residue by two novel cellulolytic fungi. Biotechnol Res Int, pp 8.

Ghose TK (1987) Measurement of cellulase activities. Pure Appl Chem 59 : 257—268.

Gauripura G, Kaliwal B (2017) Screening and optimization of β-glucosidase producing newly isolated *Lactobacillus plantarum* strain LSP-24 from colostrum milk. Biocatal Agric Biotechnol 11 : 89—96.

Himmel Xu Q, Luo Y, Ding SY, Lamed R (2010) Microbial enzyme systems for biomass conversion: Emerging paradigms. Biofuels 1 : 323—341.

Iwo JB, Manovic V, Longhurst P (2016) Biomass resources and biofuels potential for the production of transportation fuels in Nigeria. Renew Sust Energ Rev 63 : 172—192.

Ja'afarul MI, Fagade OE (2010) Optimization studies on

cellulase enzyme production by an isolated strain of *Aspergillus niger* YL128. Afr J Microbiol 4 : 2635—2639.

Jeya M, Joo AR, Lee KM, Tiwari MK (2010) Characterization of β-glucosidase from a strain of *Penicillium purpurogenum* KJS506. Appl Microbiol Biotechnol 86 : 1473—1484.

Khalili E, Huyop F, Manan FMA, Wahab RA (2017) Optimization of cultivation conditions in banana wastes for production of extracellular β-glucosidase by *Trichoderma harzianum Rifai* efficient for *in vitro* inhibition of *Macrophomina phaseolina*. Biotechnol Biotechnol Equip, pp 31.

Kocher G, Kalra K, Banta G (2008) Optimization of cellulase production by submerged fermentation of rice straw by *Trichoderma harzianum* Rut-C 8230. Internet J Microbiol, pp 5.

Kumar AK, Parikh BS (2015) Cellulose-degrading enzymes from *Aspergillus terreus* D34 and enzymatic saccharification of mild - alkali and dilute-acid pretreated lignocellulosic biomass residues. Bioresour Bioprocess 2 : 7.

Lan TQ, Wei D, Yang ST, Liu X (2013) Enhanced cellulase production by *Trichoderma viride* in a rotating fibrous bed bioreactor. Bioresour Technol 133 : 175—182.

Lehninger AL, Nelson DL, Cox MM (1993) Principles of biochemistry (1st ed). Worth Publishers, Inc.

Lynd LR, Weimer PJ, Van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: Fundamentals and biotechnology. Microbiol Mol Biol Rev 66 : 506—577.

Mukhopadhyaya S, Nandi B (1999) Optimization of cellulase production by *Trichoderma reesei* ATCC 26921 using a simplified medium on water hyacinth biomass. J Sci Ind Res 58 : 107—111.

Nathan VK, Rani ME, Rathinasamy G, Dhiraviam KN (2014) Process optimization and production kinetics for cellulase production by *Trichoderma viride* VKF3. Springer Plus 3 : 1—12.

Pandey A, Selvakumar P, Soccol CR, Nigam P (1999) Solid state fermentation for the production of industrial enzymes. Curr Sci 77 : 149—162.

Potprommanee L, Wang XQ, Han YJ, Nyobe D (2017). Characterization of a thermophilic cellulase from *Geobacillus* sp. HTA426, an efficient cellulase-producer on alkali pretreated of lignocellulosic biomass. PLoS ONE 12 : 0175004.

Raza F, Raza NA, Hameed U (2011) Solid state fermentation for the production of β-glucosidase by co-culture of *Aspergillus niger* and *A. oryzae*. Pak J Bot 43 : 75—83.

Saini A, Aggarwal NK, Yadav A (2017) Cost-effective cellulase production using *Parthenium hysterophorus* biomass as an unconventional lignocellulosic substrate. Biotech 7 : 3—12.

Sasi A, Ravikumar M, Kani M (2012) Optimization, production and purification of cellulase enzyme from marine *Aspergillus flavus*. Afr J Microbiol 3 : 1—12.

Shen Z, Zhang K, Si M, Liu M (2017) Synergy of lignocelluloses pretreatment by sodium carbonate and bacterium to enhance enzymatic hydrolysis of rice straw. Bioresour Technol 249 : 154—160.

Singhania RR, Patel AK, Sukumaran RK, Larroche C (2013) Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. Bioresour Technol 127 : 500—507.

Sørensen A, Andersen JJ, Ahring BK, Teller PJ (2014) Screening of carbon sources for beta-glucosidase production by *Aspergillus saccharolyticus*. Int Biodeterior Biodegrada 93 : 78—83.

Sørensen A, Lübeck M, Lübeck PS, Ahring BK (2013) Fungal beta-glucosidases:A bottleneck in industrial use of lignocellulosic materials.Biomolecules 3 : 612—631.

Stewart JC, Parry JB (1981) Factors influencing the production of cellulase by *Aspergillus fumigatus* (Fresenius). J Gen Microbiol 125 : 33—39.

Stutzenberger F (1990) Thermostable fungal β-glucosidases. Lett Appl Microbiol 11 : 173—178.

Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresour Technol 83 : 1—11.

Tamura K, Stecher G, Peterson D, Filipski A (2013) MEGA 6 : Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30 : 2725—2729.

Teeri TT, Koivula A, Linder M, Wohlfahrt G (1998) *Trichoderma reesei* cellobiohydrolases: Why so efficient on crystalline cellulose ? Biochem Soc Trans 26 : 173—178.

Vyas A, Vyas D, Vyas KM (2005) Production and optimization of cellulases on pretreated groundnut shell by *Aspergillus terreus* AV49. J Sci Ind Res 64 : 281—286.

Wood TM, Bhat KM (1988) Methods of measuring cellulase activities. Method Enzymol, pp 160—187.

Yoon LW, Ang TN, Ngoh GC, Chua ASM (2014) Fungal Solid-state fermentation and various methods of enhancement in cellulase production. Biomass Bioenerg 67 : 319—338.