



Original Article

Utilization of horticultural waste (Apple Pomace) for multiple carbohydrase production from *Rhizopus delemar* F₂ under solid state fermentation

Shruti Pathania*, Nivedita Sharma, Shweta Handa

Microbiology, Department of Basic Sciences, Dr. Y S Parmar University of Horticulture and Forestry, Nauni, Solan, HP 173230, India



ARTICLE INFO

Article history:

Received 10 May 2017

Received in revised form 10 August 2017

Accepted 14 October 2017

Available online 27 December 2017

Keywords:

Cellulase

Xylanase

Amylase

Pectinase

Acetone precipitation

Waste utilization

ABSTRACT

The brown rot fungus *Rhizopus delemar* F₂ was shown to produce extracellular thermostable and multiple carbohydrase enzymes. The potential of *Rhizopus delemar* F₂ in utilizing apple pomace under solid state fermentation (SSF) is the purpose of the study. Solid state fermentation (SSF) is a very effective technique opposed to submerged fermentation in various aspects. Enhanced production of multiple carbohydrases 18.20 U g⁻¹ of cellulase, 158.30 U g⁻¹ of xylanase, 61.50 U g⁻¹ of pectinase and amylase 21.03 U g⁻¹ was released by microwave pretreatment of apple pomace at 450 W for 1 min and then by incubation the culture thus obtained at 30 °C for 6 days with moisture content of 1:4.5. Apple pomace can serve as a potential source of raw material for the production of multiple carbohydrases. Besides, it can find great commercial significance in production of bioethanol and various industries like textile, fruit juice, paper and pulp industry.

© 2017 Production and hosting by Elsevier B.V. on behalf of Academy of Scientific Research & Technology. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Enzymes of industrial interest are routinely being explored in various microbial hosts to increase the yield, to satisfy the needs of both the manufacturer and the end user. Microbes serve as a producer a variety of enzymes that have been successfully used on industrial scale, [1,2]. Microbial enzymes have found their applications in textile (Amylase, Cellulose and Oxidoreductase); detergents (Protease, Lipase and Cellulase); paper (Xylanase and Lipase); food (Pectinase, Protease and Cellulase) and leather (Protease and Lipase) industries. In recent years, the potentials of using fungi as biotechnological sources of industrially-relevant multiple carbohydrases have stimulated interest in the exploration of extra-cellular enzyme producing microbes [3]. Fungi are of great interest as a source of multiple carbohydrases due to their easy cultivation and high production of enzymes [4].

A state like Himachal Pradesh has vast cultivable area and due to favorable climate, it is actively involved in horticulture and farming and is thus renowned as the “fruit bowl of India”. Apple,

which is the main fruit crop of the state is cultivated over an area of 9.97 thousands hectare resulting in approximately 5.8 Lac tones of apple production annually [5]. Tonnes of apple produce are processed annually in the state and as a result, 1750 ton of apple pomace is disposed of into the environment every year on an average. In the absence of a technology that can recycle/utilize this waste, apple pomace is posing a serious hazard to the environment [6].

Therefore, there is an urgent need for developing a viable technology that can utilize apple pomace as a raw material for production of bioethanol. The ability of certain microorganisms to produce multiple carbohydrases such as cellulose, hemicelluloses, starch, pectin biomass can be used to generate resources for a greener and cleaner process that can produce simple sugars (pentoses and hexoses) from a readily available and cheap biomass i.e., apple pomace. Solid state fermentation is an attractive method for multiple enzyme production, especially for fungal cultivations, because of its high productivity per reactor volume and its cost effective nature [7,8]. The physical support and the energy required for the growth of fungus and desirable metabolite production is primarily provided by the substrate and also for reducing the cost of enzyme, selection of cheap and easily available substrate appears to be essential. Therefore it's important to select a desirable substrate for solid state fermentation [9]. The hyphal

Peer review under responsibility of National Research Center, Egypt.

* Corresponding author.

E-mail address: shrutipathania89@gmail.com (S. Pathania).

mode of growth gives the filamentous fungi the power to penetrate into the solid substrates and simplifying the structure of substrate. The hydrolytic enzymes are excreted at the hyphal tip, without large dilution like in the case of SS, that makes the action of hydrolytic enzymes very efficient and allows penetration into most solid substrates. Penetration increases the accessibility of all available nutrients within particles [10].

This paper describes the selection of *Rhizopus delemar* F₂ as a potential producer of different multiple carbohydrase and further optimization of process parameters for enhancing multiple carbohydrases production under solid state fermentation.

2. Materials and methods

2.1. Microorganism

Samples in the form of cow dung, compost and wood bark were collected from the area nearby Solan. These samples were taken in the laboratory and further processing was done to isolate fungi capable of producing various depolymerizing enzymes. Samples were enriched with 100 mL of Erlenmeyer flasks supplemented with 0.5% (w v⁻¹) i.e., cellulose, xylan and pectin and incubated at 30 °C for 4 days. The fungus was further isolated on Cookes Red Bengal medium [11] with an initial pH of 5.5 and incubated at 30 °C for 7 days. The pure culture was obtained and maintained at 4 °C in potato dextrose agar medium. To obtain an inoculum of the fungal culture, a 4 mm diameter sample of mycelium was punched out from the plate and placed for examination.

2.2. Morphology examination of F₂

Strain F₂ was cultivated on a plate of Cookes red Bengal medium incubated at 30 °C in the dark for 7 days. The filamentous fungi were identified to the genus level based on macroscopic and microscopic characterization [13]. Colony morphology was identified using light microscope (image analyzer).

2.3. Molecular analysis

The genomic DNA extracted from approximately 100 mg of freeze-dried fungal mycelia by crushing in 1.5 mL microcentrifuge tubes using micropestles. AP1 Buffer of 400 µl and 4 µl of RNase, a stock solution (100 mgmL⁻¹) were added to a maximum of 100 mg (wet weight) or 2 mg (dried) disrupted fungal tissue and vortex vigorously. The mixture was incubated for 10 min at 65 °C and mixed 2 or 3 times during incubation by inverting tube. 130 µl buffer was added to the lysate and incubated for 5 min on ice. Lysate was centrifuged for 5 min at 20,000g. After centrifugation supernatant was applied to QIAshredder Mni spin column and centrifuged for 2 min at 20,000g. Flow through fraction obtained from above step was placed into a new tube without disrupting the cell debris pellet. AP3 E⁻¹ buffer of 1.5 Vol was added to the cleared lysate and properly mixed by pipetting. 650 µl of the mixture so obtained from above step was kept into the DNeasy mini spin column placed in a 2 mL collection tube followed by centrifugation for 1 min at 6000g. flow through so obtained was discarded. Place the DNeasy Mini spin column into a new 2 mL collection tube and of 500 µl buffer AW was added, followed by centrifugation for 1 min at ≥6000g. Flow through was discarded and collection tube was reused in next step. AW buffer of 500 µl was added to the DNeasy Mini spin column followed by centrifugation for 2 min at 20,000g to the dry membrane. DNeasy Mini spin column containing sample was placed to a 1.5 mL or 2 mL microcentrifuge tube and 100 µl of buffer AE was added onto the DNeasy membrane. Sample was incubated for 5 min at room temperature (15–25 °C)

and then centrifugation for 1 min at 6000g and flow through so obtained was discarded to elute the DNA. PCR amplification was done to confirm the identity of the fungal strain F₂, the small sub-unit 5.8 SrRNA genes were amplified from the genomic DNA with (ITS-1-5' TCCGTAGGTGAACCTGCGG 3') and (ITS-4-5' TCCTCCGCTTATTGATATGC-3') primers to get an amplicon size of 1500 bp. The amplified PCR product was cleaned up using PCR clean up kit (Real Genomics Hi yieldTM Make [14].

2.4. Collection of substrate

Dried apple pomace was collected from Himachal Pradesh Horticulture Produce Marketing and Processing Corporation limited (HPMC) Prawnoo and was sun dried and stored in air tight containers for compositional analysis.

2.5. Proximate chemical composition analysis of the substrate

The chemical composition of apple pomace was analysed for cellulose, hemicelluloses and lignin following the Technical Association of Pulp and Paper Industry (TAPPI) protocols, (extractives-TAPPI Method T6m-59 [15]; holocellulose-TAPPI Method T9m-54 [15] lignin-TAPPI Method T12m-59 [15] and starch and pectin by following procedure of Sadasivam and Manickam [16].

2.5.1. Inoculum preparation

In present study, conidial inoculum and spore suspension was prepared by adding 10 mL of sterile distilled water into a 7 days old slant culture aseptically. The plate was scratched with the loop, the mat was scratched and added into the flask containing apple pomace.

2.5.2. Screening of fungus based on the capability to utilize apple pomace for enzyme production under SSF

1 mL of each spore and conidial suspension were transferred to each 5 Erlenmeyer flasks (500 mL) containing 5 g of apple pomace and 10 mL of moistening agent modified BSM (composition: 6.0 g Na₂HPO₄, 3.0 g KH₂PO₄, 0.5 g NaCl, 1.0 g NH₂Cl and separately sterilized solution of 1 M MgSO₄ (2 mL) and 1 M CaCl₂ (0.1 mL) was added after the medium was autoclaved with fixed ratio of 1:2 i.e., substrate: moisture.

2.5.3. Enzyme extraction

After 7 days of incubation, 50 mL of phosphate buffer (0.1 M, pH 6.9) was added to the flasks and kept under shaking for 1 h. The flask contents were filtered using muslin cloth and the process was repeated twice. The filtrate was centrifuged at 12000 rpm for 15 min at 4 °C and the supernatant was collected for further studies [17].

2.6. Analytical methods

2.6.1. Enzyme assays

2.6.1.1. FPase activity. The reaction mixture contained 0.5 mL of 1% of Carboxymethylcellulose (CMC) in citrate buffer (0.055 M, pH 5) and 0.5 mL of diluted enzyme (supernatant). Reaction mixture was incubated at 50 °C for 30 min. After incubation 3 mL of DNSA reagent was added. Tubes were immersed in boiling water bath and removed after 15 min when color development was complete. Control was run with all the components except the enzyme. Tubes were cooled at room temperature and O.D was read at 540 nm in spectrophotometer against the reagent blank i.e., 1 mL of distilled water and 3 mL of DNSA reagent. The standard curve was made from the stock solution of glucose (0.4 mg mL⁻¹). The enzyme activity was expressed in terms of International Unit (IU) as described by Reese and Mendel [18].

2.6.1.2. Xylanase activity. To 0.5 mL of xylan solution (which was incubated overnight at 37 °C centrifuged and clear supernatant was used, 0.3 mL citrate buffer (pH 5.5) was added and 0.2 mL of enzyme. The control was run with all the components except the enzyme. The reaction mixture was incubated at 45 °C for 10 min. After the incubation, 3 mL of DNSA reagent was added and the mixture was then heated on boiling water bath for 30 min. After cooling down at room temperature, absorbance of reaction mixture was read at 540 nm. The enzyme activity was expressed in terms of International Unit (IU) method by Miller [19].

2.6.1.3. β -Amylase assay. To 0.5 mL of enzyme solution which was incubated with 0.2% starch at 37 °C for 15 min 3 mL of DNSA reagent was added to it and the mixture was heated on boiling water bath for 15 min. After cooling down to room temperature, absorbance of reaction mixture was read at 540 nm. The standard curve was made from the stock solution of glucose (0.4 mg mL⁻¹). The enzyme activity was expressed in terms of International Unit (IU) a method by Miller [19].

2.6.1.4. Pectinase assay. To 1 mL of pectin that was dissolved in 0.1 mL of citrate buffer (0.6 M, pH 6) and 1 mL of culture supernatant was added. Reaction mixture was incubated at 35 °C for 30 min. After incubation 3 mL of DNSA reagent was added. Tubes were immersed in boiling water bath and removed after 15 min when colour development was complete. Control was run with all the components except the enzyme. Tubes were cooled at room temperature and O.D was read at 540 nm in spectrophotometer against a reagent blank i.e., 1 mL of distilled water and 3 mL of DNSA reagent. The standard curve was prepared from the stock solution of glucose (0.4 mg mL⁻¹) a method by Miller [19].

2.6.2. Protein determination

Total protein content was determined by the method as described by Lowry [20].

2.7. Effect of different parameters on multiple carbohydrases production under SSF

2.7.1. Effect of different microwave doses

To 5 g of each untreated biomass i.e., apple pomace was exposed to different microwave doses (watt) i.e., 150, 300, 450, 600 and 900 W for 1 min in microwave oven and then adding 17.50 mL of moistening agent viz. Basal salt medium (pH 5.5) substrate: moistening agent (1:3.5) in 250 mL Erlenmeyer flask and autoclaved. After the production of multiple carbohydrases under SSF for 7 days, fungal hyphae were taken from the flask for inoculation into the broth of potato dextrose agar (PDA) and incubated for 5 days for the formation of hyphae, thereby the purity of the fungal culture was checked at stages of multiple carbohydrases production.

2.7.2. Effect of moisture ratio

To 5 g of each 450 watt microwave pretreated biomass, 17.5 mL of moistening agent viz. basal salt medium was added (in the ratio of 1:3.5 i.e., substrate: moistening agent) in 250 mL of Erlenmeyer flask and further autoclaved at 121 °C. After autoclaving, the flasks were inoculated with 10 mL of *Rhizopus delemar* F₂ having 1 × 10⁷ spores mL⁻¹ and incubated at different moisture ratio ranging from 1:3:0, 1:3:5, 1:4:5, 1:5:5 and 1: 6:5 for 7 days in static phase.

2.7.3. Effect of incubation time

To 5 g of each 450 watt microwave pretreated biomass, 22.5 mL of moistening agent viz. basal salt medium was added (in the ratio of 1:4.5 i.e., substrate: moistening agent) in 250 mL Erlenmeyer flask and autoclaved further flasks were incubated for a varied time periods (1, 2, 3, 4, 5, 6, 7 and 8 days), the culture contents were centrifuged at 10,000 rpm for 15 min at 4 °C. Supernatants were collected and stored at 4 °C in refrigerator for further use. The quantitative tests were performed with the supernatant.

2.7.3.1. Statistical analysis. Treatment effects were compared with a least significant difference method using completely randomized design (CRD).

3. Results and discussion

3.1. Isolation and screening of fungi producing multiple carbohydrase

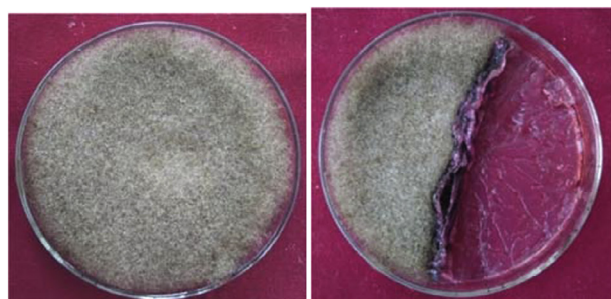
After extensive screening of fungal biodiversity from various sites of Solan, a total of 5 isolates were isolated on the basis of their ability to hydrolyze apple pomace under SSF. All the isolates were tentatively identified on the basis of macroscopic and microscopic examination of the colonies and belonged mainly to *Aspergillus*, *Fusarium*, *Penicillium* and *Rhizopus* sp. with aseptate brownish mycelium, conidia were rough, globular and tightly packed (Table 1). Screening of fungal isolates showed that the fungus *Rhizopus* sp F₂ was capable of yielding maximum multiple carbohydrase under solid state fermentation after incubation for 7 days at 35 °C using basal salt medium of pH 5.5 moistened with 1:4.5 of moisture solution. Fungi as a saprophytic ability expressed by fast mycelia growth, spore production, presence of efficient and extensive system of powerful enzymes are capable to utilize complex chemical substances as energy source and therefore make them important in nature. The *Rhizopus* sp.F₂ was capable of forming a mat on the cooked red beal medium that leaves the patch on the medium as shown in Fig. 1.

The microscopic analysis of the colonies resembles with *Rhizopus* sp. (Fig. 1). The 5.8S rRNA of F₂ was successfully (Fig. 2) and the aligned sequences of this amplified DNA from F₂ was submitted to GenBank for homology searching against the GenBank or the proprietary fungal DNA database, the sequence of F₂ was found to share 99% with those of *Rhizopus delemar* F₂ (NCBI KX512312) as depicted in alignment view and distance. A phylogenetic relationship was established through the alignment and cladistic analysis

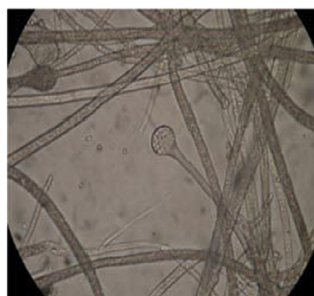
Table 1

Isolation of multiple carbohydrase-producing fungi from different sites and their morphological and cultural characteristics.

| Fungal Isolate | Source | Mycelium | Spore | | |
|----------------|---------------|---------------|--------|----------|--------------------------|
| | | | Colour | Texture | Tentative Identification |
| F ₁ | Compost | Hyphae (long) | White | Rough | <i>Rhizopus</i> sp. |
| F ₂ | Decaying wood | Short hyphae | Brown | Feathery | <i>Rhizopus</i> sp. |
| F ₃ | Cow dung | Short hyphae | Brown | Rough | <i>Trichoderma</i> sp. |
| F ₄ | Compost | Filamentous | White | Feathery | <i>Phytium</i> sp. |
| F ₅ | Compost | Short Hyphae | Black | Rough | <i>Aspergillus</i> sp |



(a) (b)



(c)



(d)

Fig. 1. (a and b) Colonies and mat formation by *Rhizopus* sp. F₂ (4 days) c) Conidiospore of *Rhizopus* sp. under light microscope, d) colonization of *Rhizopus* sp. F₂ on apple pomace (7th day).

of homologous nucleotide sequence among these fungal species Fig. 3.

3.2. Composition of apple pomace

The composition of the untreated substrate, collected in a single batch, was analyzed and reported as dry weight of major components i.e., cellulose, lignin, hemicelluloses, pectin and starch holocellulose i.e., cellulose + hemicellulose content i.e., cellulose 36%, hemicelluloses of 16.6%, Pectin 11% and starch of 8.6% but more lignin content of 19% which probably makes its degradation comparatively difficult (Table 2). Cellulose, hemicelluloses, pectin and starch are important constituents of apple pomace as these are responsible to yield fermentable sugars upon hydrolysis of carbohydrates. The fermentable sugars thus produced can further be fermented into bio-ethanol using specific yeast and/or bacteria. The

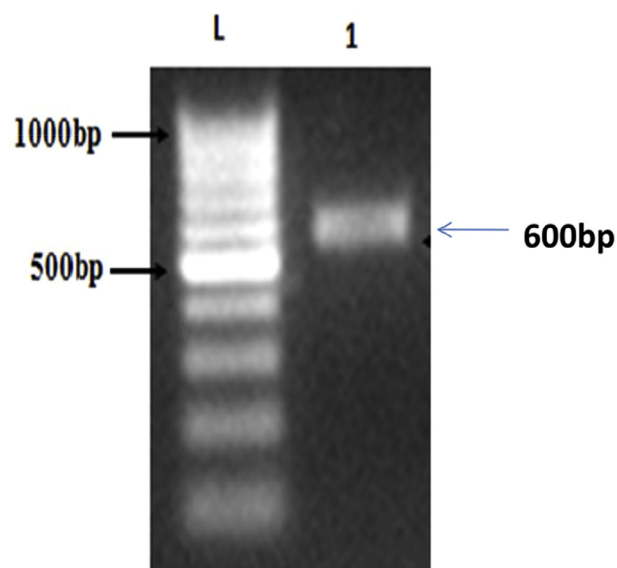


Fig. 2. Molecular identification of screened fungal isolate by ITS 5.8S rRNA gene technique (L = Ladder, Lane 1 = *Rhizopus* sp. F₂).

results derived in this experiment prove that it can serve as a rich source for its bioconversion into value added products viz. ethanol (see Table 3).

3.3. Selection and identification of fungus

In the present study, major horticultural waste i.e., apple pomace was used for screening of fungus capable of utilizing the substrate for multiple carbohydrase production under solid state fermentation as shown in Fig. 4. The maximum multiple carbohydrase activity of 7.71 U g⁻¹ cellulase, 92.42 U g⁻¹ of xylanase, 29.62 U g⁻¹ of Pectinase and 7.30 U g⁻¹ of amylase was achieved with by F₂ followed by *Trichoderma* sp. F₃ with a yield of 7.21 Cellulase, 98.00 U g⁻¹ of xylanase, 26.60 U g⁻¹ of pectinase and 5.51 U g⁻¹ of amylase recommending the use of *Rhizopus* sp.F₂ to utilize environmental waste and secreting potential enzymes.

The fungal isolate capable of producing highest multiple enzymes were identified by using 5.8 S rRNA and accession KX512312.

3.4. Optimization of cultural conditions and medium composition for the production of xylanase by *Rhizopus delemar* F₂

3.4.1. Effect of microwave dose

Pretreatment is one of the important parameters that estimates the success of SSF system, as it disturbs the crystallinity of the structure and exposes more surface for biodegradation [21]. Therefore, the effect of different doses of microwave on multiple carbohydrase production by *Rhizopus delemar* F₂ was examined and the results obtained are shown in Fig. 5. The production of different synergistic enzymes were found maximum at 450 watt i.e., 11.90 U g⁻¹ of cellulose, 150.23 U g⁻¹ of xylanase, 12.0 U g⁻¹ of amylase and pectinase yield of 52.50 U g⁻¹ incubation at ambient temperature (28 ± 3 °C) after incubation for 7 days, with the increase in microwave dose (600 watt) the temperature rises which might affect the structure of the substrate in terms of its binding with the enzyme, thereby tempering the enzyme activity (11.90 U g⁻¹ of cellulose, 150.23 U g⁻¹ of xylanase, 48.90 U g⁻¹ of pectinase and 15.50 U g⁻¹ of amylase).

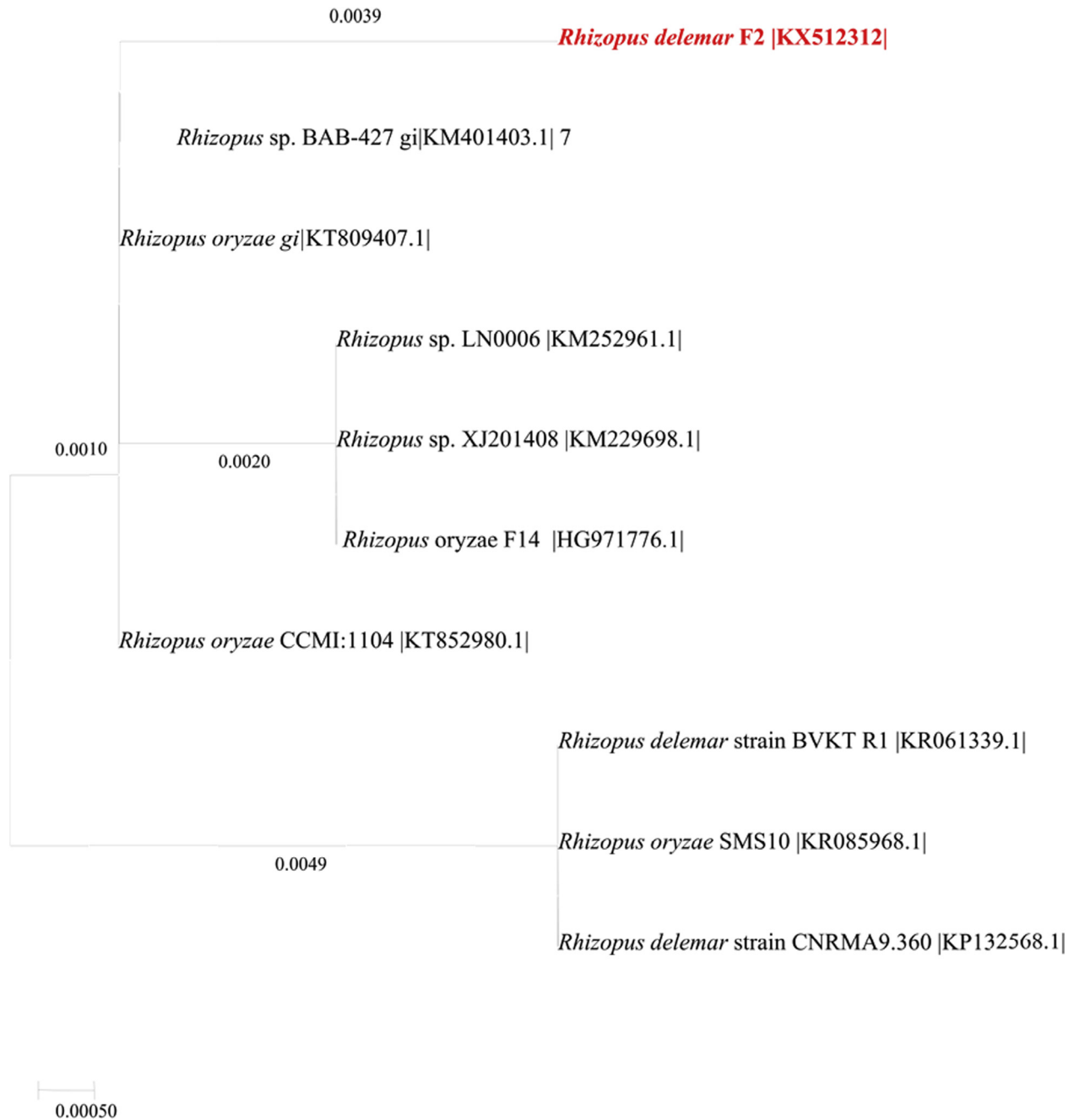


Fig. 3. Neighbor-joining tree with 1000 bootstrap values in MEGA 7.0 showing phylogenetic relationship of *Rhizopus delemar* F₂ based on a distance matrix analysis of 5.8S rRNA sequences.

Table 2
The composition analysis of apple pomace.

| Apple pomace | Content |
|---------------|---------|
| Cellulose | 36% |
| Hemicellulose | 11% |
| Lignin | 19% |
| Pectin | 16.6% |
| Starch | 3.6% |
| pH | 4.0 |

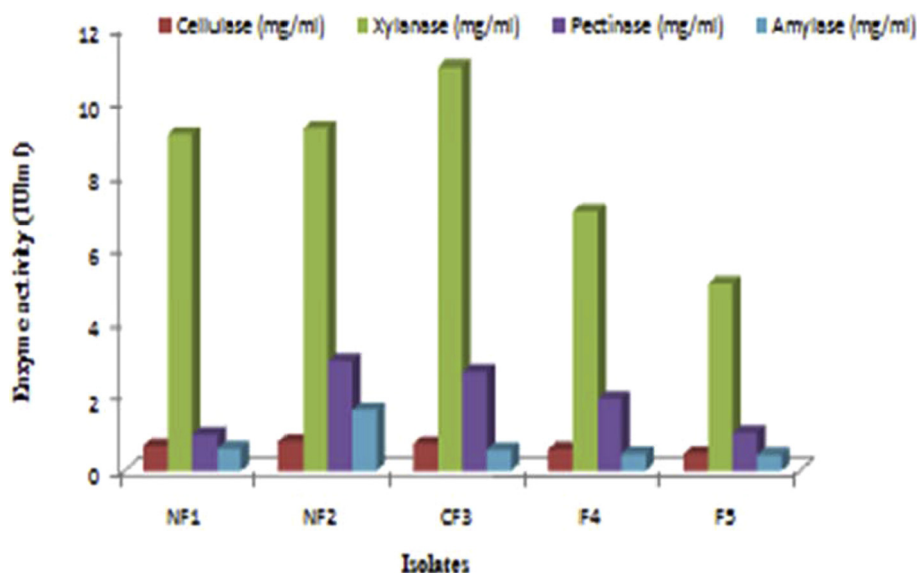
Microwave irradiation is reported to change the crystalline/microfibril structure of cellulose, degrade lignin and hemicellulose in the substrate and increase enzymatic susceptibility [22]. Com-

pared to conduction/convection heating, microwave directly interacts between a heated target and electromagnetic field to generate heat [23,24]. Therefore, heating is volumetric and rapid method used to treat the substrate with its unique heating feature has in an 'explosion effect' among particles and improves disruption of recalcitrant structure of lignocelluloses. In addition, the electromagnetic field used in the microwave might cause non-thermal effects that also accelerate destruction of crystal line structures [25]

Pathania et al. [26] provided a microwave pretreatment to the rice straw for effective production of cellulase i.e., 43.07 U g⁻¹ and xylanase of 281.07 U g⁻¹ at 50 °C for 8 days by *M.thermophila* SH1 under SSF. Similarly, Nomanbhay [27] enhanced the enzyme production using microwave irradiation enzyme coupling catalysis

Table 3Purification stages of multiple carbohydrase by *Rhizopus delemar* F₂.

| 3a Cellulase | | | | | | | |
|-----------------------|-------------------|---|--|--------------------------------------|--------------------------------|---------------------------------|-----------------------------|
| Purification Step | Total volume(mL) | Cellulase activity (U g ⁻¹) | Total activity (IU mL ⁻¹) | Total protein (mg mL ⁻¹) | Specific activity [*] | Purification fold ^{**} | Recovery (%) ^{***} |
| Crude | 100 | 18.2 | 182 | 1.52 | 1.19 | 1 | 100 |
| Acetone precipitation | 25 | 23.4 | 58.5 | 0.98 | 2.38 | 2 | 64.46 |
| 3.b Xylanase | | | | | | | |
| Purification step | Total volume (ml) | Xylanase Activity (U g ⁻¹) | Total activity (IU mL ⁻¹) | Total protein (mg mL ⁻¹) | Specific activity [*] | Purification fold ^{**} | Recovery (%) ^{***} |
| Crude | 100 | 158.3 | 1523 | 1.52 | 10.01 | 1 | 100 |
| Acetone precipitation | 25 | 163.3 | 408.25 | 0.98 | 16.66 | 1.66 | 64.46 |
| 3c. Pectinase | | | | | | | |
| Purification step | Total volume (ml) | Pectinase activity (U g ⁻¹) | Total activity (IU mL ⁻¹) | Total protein (mg mL ⁻¹) | Specific activity [*] | Purification fold ^{**} | Recovery (%) ^{***} |
| Crude | 100 | 61.50 | 745 | 1.52 | 4.90 | 1 | 100 |
| Acetone precipitation | 25 | 9.05 | 226.25 | 0.98 | 9.23 | 1.88 | 64.46 |
| 3d. Amylase | | | | | | | |
| Purification step | Total volume (ml) | Amylase Activity (U g ⁻¹) | Total activity [*] (IU mL ⁻¹) | Total protein (mg mL ⁻¹) | Specific activity [*] | Purification fold ^{**} | Recovery (%) ^{***} |
| Crude | 100 | 21.0 | 210 | 1.52 | 1.38 | 1 | 100 |
| Acetone Precipitation | 25 | 4.04 | 101 | 0.98 | 4.12 | 2.98 | 64.46 |

^{*} Specific activity = Enzyme Activity/mg protein concentration.^{**} Purification fold is increase in specific activity.^{***} Percent recovery is remaining protein concentration as% of the initial protein concentration.**Fig. 4.** Screening of fungal isolates for multiple carbohydrase production.

on oil palm empty fruit bunch. Hu et al. [28] observed lignin removal in 63–70% of substrate with various particle size ranges of 1.0–2.0, 0–0.5, 0.5–0.25, and <0.25 mm of switchgrass with microwave-assisted alkali pretreatment but the energy was intensive, thus increased the pretreatment cost (see Fig. 6).

3.4.2. Effect of moisture

Solid substrates used were insoluble in water, therefore water will have to be absorbed onto the substrate particles, which can be used by the microorganisms for growth and metabolic activity [28]. Water causes the swelling of the substrate and facilitates uti-

lization of substrates by the fungus as making the substrate by providing the adequate moisture for growth of fungus. Increasing moisture level is believed to have reduced the porosity of substrate, thus limiting the oxygen transfer into the substrate [29,30].

Every microorganism has a distinct water activity requirement essential for its growth in the solid state fermentation. Different experiments were performed by changing the amount of diluents and keeping solid ratio constant. As indicated in Fig. 3, the enzymes production was optimum using the Basal salt medium (BSM) which was moistened with moistening agent in the ratio of 1:4.5 with the production of 15.90 U g⁻¹ of cellulose, 152.50 U g⁻¹ of

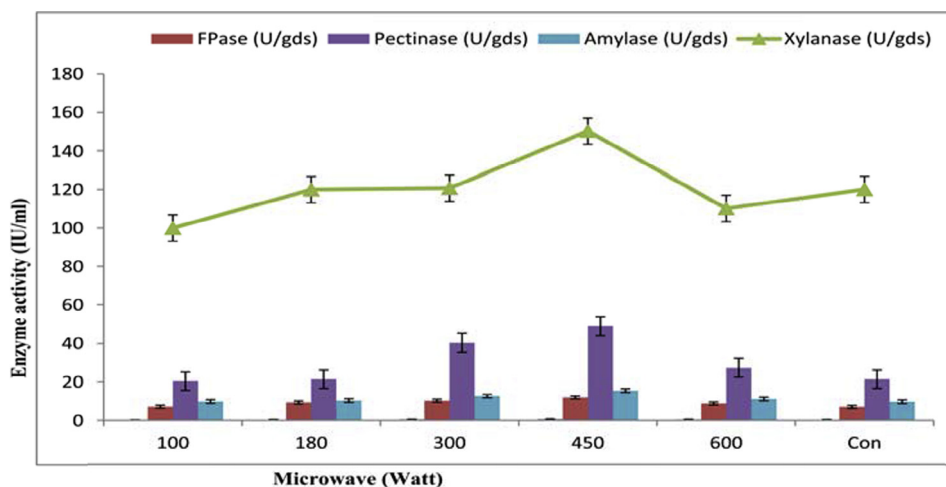


Fig. 5. Effect of microwave pretreatment on multiple carbohydrase production from *Rhizopus delemar* F₂.

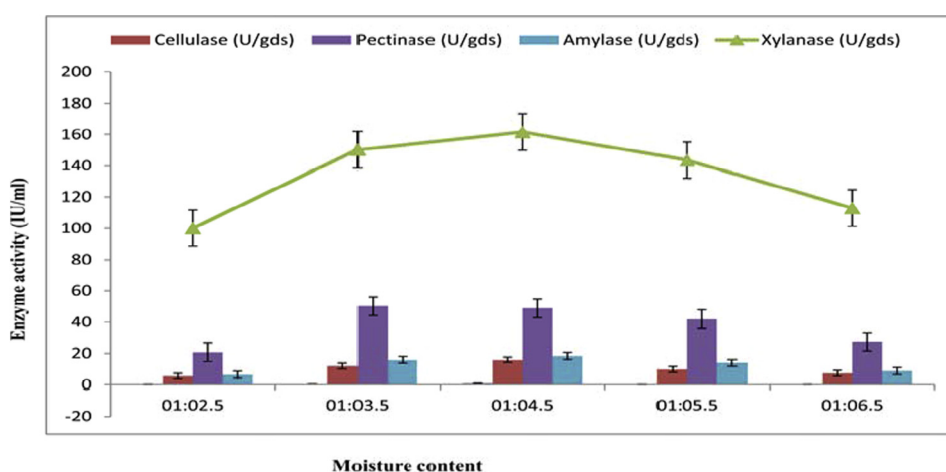


Fig. 6. Effect of moisture on microwave pretreated on multiple carbohydrase from *Rhizopus delemar* F₂.

xylanase, 51.09 U g⁻¹ of pectinase and 18.40 U g⁻¹ of amylase whereas increase in moistening agent doesn't leads to decreased enzyme activity with 7.4 U g⁻¹ cellulase, 112.9 U g⁻¹ of xylanase, 27.4 U g⁻¹ of pectinase and 8.8 U g⁻¹ of amylase with 1:6.5 of moistening agent. In SSF the optimal moisture content depends on the requirement of microorganisms, type of the substrate and the types of end products. The high moisture level increases the free excess liquid in the medium which ultimately decrease in growth and enzyme production.

Dutt and Kumar [31] optimized the moisture ratio of 1:3 (substrate: moistening agent) for enhanced cellulose production under solid state fermentation using *Aspergillus flavus* (AT-2) and *Aspergillus niger* (AT-3). Similarly Ghoshal et al. [32] observed that the maximum yield of xylanase was observed when lignocellulosic agrowaste was moistened in 1:5 for 5 days of incubation.

3.5. Effect of incubation days

Xylanase production was checked by incubating the inoculated flasks for various time periods and it was noted that enzyme production was gradually increased with increase in fermentation period and maximum production was achieved after 10th days of fermentation period as shown in Fig. 7. As the fermentation period

was increased, a decrease in enzyme production was observed. The time taken to achieve maximum enzymes production after 6 days of incubation at 30 °C giving the enzymatic yield of 18.20 U g⁻¹ of cellulase, 158.30 U g⁻¹ of xylanase, 61.50 U g⁻¹ of pectinase and 21.01 U g⁻¹ of amylase at 30 °C using apple pomace with 1:4.5 of moisture ratio whereas decrease in enzyme activity after increase in incubation day further on 8th day i.e., 11.60 U g⁻¹ of cellulase, 113.60 U g⁻¹ of xylanase, 50.20 U g⁻¹ of pectinase and 18.04 U g⁻¹ of amylase as depicted in Fig. 4. The growth rate of microorganism and its enzyme production pattern depends upon the duration needed for incubation day [33]. The enzyme production of the fungal grown on the 5 day in the present study indicates that the maximum enzyme might have been produced either in the exponential growth phase, as during these phase growth elements such as amino acids, nitrogen, vitamins, protein and enzymes required for essential metabolic processes or stationary phase are being synthesized by the fungus.

Okafor et al. [34] reported a xylanase producing fungal strain *Penicillium chrysogenum* PCL501 isolated from wood-wastes containing different carbon sources (oat spelts xylan, wheat bran, sawdust and sugarcane pulp. Media containing sugarcane pulp gave a peak value of 1.39 U mL⁻¹ at 144 h of fermentation obtained using sugarcane as a substrate.

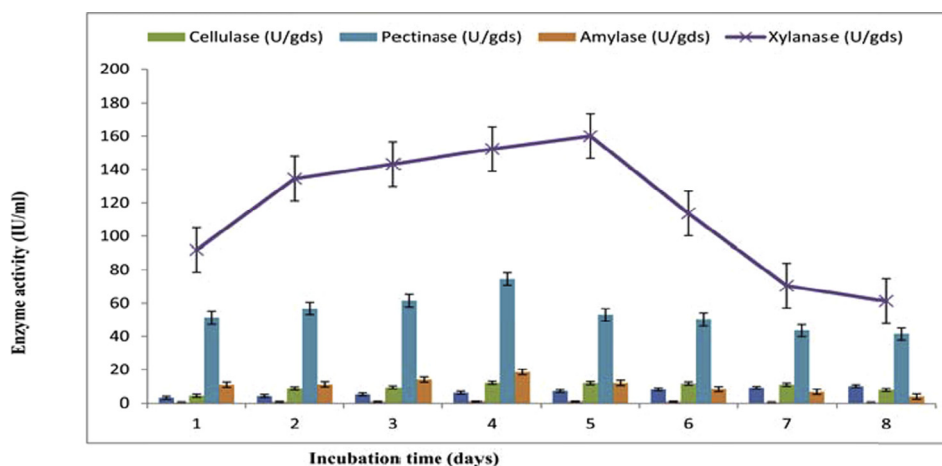


Fig. 7. Effect of Incubation time on multiple carbohydrase from *Rhizopus delemar* F₂.

4. Conclusions

This strain (*Rhizopus delemar* F₂) had the potential to utilize lignocellulosic waste, such as sugarcane bagasse, as a carbon source to produce valuable enzymes, thus reducing enzyme production cost. Pretreatment of the substrate plays a pivotal role in enzyme production due to the increased accessibility of nutrients to the fungus hindered by thick hard layer of lignin. Optimization of process parameters is a pre-requisite to enhance the yield, which is very helpful in large-scale production. Therefore, recommending an appreciable way of utilizing a substrate i.e., apple pomace for multiple carbohydrase production and therefore serve as an alternative source for better utilization of environmental waste i.e., apple pomace.

Acknowledgement

The author are very thankful to the Department of Basic Sciences, Microbiology section for funding this study were the major part of the experiment were conducted.

Funding

The author are very thankful to the Department of Basic Sciences, Microbiology section for funding this study were the major part of the experiment were conducted.

Conflict of interest

Author Dr. Shruti Pathania, Dr. Nivedita Sharma and Dr. Shweta Handa has received grant from State council of Environment Science and Technology, Government of Himachal Pradesh, India. aThe authors declare that they have no conflict of interest.

References

- [1] Panda SS, Sahoo K, Das R, Dhal NK. Pectinolytic and cellulolytic activity of soil fungal isolates from simlipal bioreserve forest. *World Environ* 2012;2(2):1–3.
- [2] Sohail M, Naseeb S, Sherwani SK, Sultana S, Aftab S, Shahzad S, Ahmad A, Khan SA. Distribution of hydrolytic enzymes among native fungi: *Aspergillus* the predominant genus of hydrolase producer. *Pak J Bot* 2009;41:2567–82.
- [3] Akpan I. Screening for novel fungal biocatalysts, *A Niger. J Microbiol* 2004;18:288–92.
- [4] Guimaraes LHS, Peixoto-Nogueira SC, Michelin M, Rizzatti ACS, Sandrim VC, Zanoelo FF, Aquino ACMM, Junior AB, de Lourdes Maria, Polizeli AB. Screening of filamentous fungi for production of enzymes of biotechnological interest. *Braz J Microbiol* 2006;37:474–80.
- [5] FAO. FAOSTAT. The United Nations: Food and Agriculture Organisation; 2012.

- [6] Almeida MMB, Sousa PHM, Arriaga AMC, Prado GM, Carvalho M, Emanuel C, et al. Bioactive compounds and antioxidant activity of fresh exotic fruits from northeastern Brazil. *Food Res Int* 2011;44:2155–9.
- [7] Purkharthofer H, Sinner M, Steiner W. Cellulase-free xylanase from *Thermomyces langinosus* optimization of production in submerged and solid state culture. *Enzyme Microb Technol* 1993;15:677–82.
- [8] Pandey A, Seelvakumar P, Soccol CR, Nigam P. Solid state fermentation for the production in industrial important enzyme. *Enzyme Microb Technol* 1999;15:677–82.
- [9] Pandey A. Solid state fermentation. *Biochem Eng J* 2003;36:1–4.
- [10] Raimbault M. General and microbiological aspects of solid substrate fermentation. *Electron J Biotechnol* 1998;1:1–15.
- [11] Cooke. Antibiotics, *Chemother* 1957;4:657.
- [12] Raper KB, Fennell DI. The genus *Aspergillus*. Baltimore Williams and Wilkins Company; 2001. p. 686.
- [13] Zhao X, Zhou Y, Zheng G, Liu D. Microwave pretreatment of substrates for cellulase production by solid-state fermentation. *J Appl Biochem Biotechnol* 2010;160:1557–71.
- [14] Anonymous. Alcohol benzene solubility of wood. Official standard T6m–59. Technical Association of Pulp and Paper Industry. New York; 1959.
- [15] Berghem LER, Pettersson LG. Mechanism of enzymatic cellulose degradation and purification of a cellulolytic enzyme from *T. viride* active on highly ordered cellulose. *J Biochem* 1937;37:21–30.
- [16] Sadasivam S, Manickam A. Biochemical methods for agricultural science. New Age international Publishers, 2nd ed. New Delhi; 1996. p. 772.
- [17] Reese ET, Mandel M. Enzymatic hydrolysis of cellulose and its derivative. In: Whistler RL, editor. *Methods of carbohydrate chemistry*. London: Academic Press; 1963. p. 139–43.
- [18] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal Chem* 1959;31:426–8.
- [19] Lowry OH, Rosebrough NJ, Faer AL, Randall RJ. Protein measurement with folin phenol reagent. *J Biol Chem* 1951;193:265–75.
- [20] Ethais S, Omar RR, Kamal SMM, Blak DRA. Microwave-assisted pretreatment of lignocellulosic biomass: a review. *JESTEC* 2015;3:97–109.
- [21] Singh R, Tiwari S, Srivastava M, Mina U. Effect of combination of microwave and Hydrogen Peroxide (H₂O₂) pretreatment on enzymatic saccharification of rice straw. *Int J Environ Eng Manage* 2013;4:529–42.
- [22] Ooshima H, Aso K, Harano YY, Yamamoto I. Microwave treatment of cellulosic materials for their enzymatic-hydrolysis. *Biotechnol Lett* 1984;6:289–94.
- [23] Azuma JL, Tanaka F, Koshijima T. Enhancement of enzymatic susceptibility of lignocellulosic wastes by microwave irradiation. *J Ferment Technol* 1984;62:377–84.
- [24] Verma A, Kumar S, Jain PK. Key pretreatment technologies on cellulosic ethanol production. *J Sci Res* 2011;55:57–63.
- [25] Pathania S, Sharma N, Kumar S. Utilization of microwave irradiated rice straw as a substrate for cost effective production of cellulase and xylanase from a potential fungus *Myceliophthora thermophila* SH1 isolated from hot spring of Northern Himalayans. *Arc Appl Sci Res* 2015;7:9–15.
- [26] Nomanbhay SM, Hussain R, Palanisamy K. Microwave-Assisted alkaline pretreatment and microwave assisted enzymatic saccharification of oil palm empty fruit bunch fiber for Enhanced fermentable sugar yield. *J Sustain Bioenergy Syst* 2015;3:7–17.
- [27] Hu Z, Wang Y, Wen Z. Alkali (NaOH) pretreatment of switchgrass by radio frequency-based dielectric heating. *J Appl Biochem Biotechnol* 2008;148:71–81.
- [28] Pandey A, Soccol CR, Nigam P, Soccol VT. Biotechnological potential of agro-industrial residues I: sugarcane bagasse. *Bioresource Technol* 2000;74:69–80.
- [29] Raimbault M, Alazard D. Culture method to study in fungal growth insolid state fermentation. *Appl Microbial Biotechnol* 1980;9:199–209.

- [31] Patil NP, Chaudhari BL. Production and purification of pectinase by soil isolate *Penicillium* sp and search for better agro residue for its SSF. Recent Res Sci Technol 2002;2:36–42.
- [32] Dutt D, Kumar A. Optimization of cellulase production under solid-state fermentation by *Aspergillus flavus* (at-2) and *Aspergillus niger* (at-3) and its impact on stickies and ink particle size of sorted office paper. Cellul Chem Technol 2014;48:285–98.
- [33] Ghoshal G, Banerjee UC, Shivhar VS. Utilization of agrowaste and xylanase production in solid state fermentation. J Biochem Tech 2015;6:1013–24.
- [34] Topkas E, Moukaouli M, Dimarogona G, Christakopoulos P. Expression characterization and structural modeling of feruloly esterase from the thermophilic fungus *M.thermophila*. Proc Biochem 2003;38:1539–43.

Further reading

- [12] Teather RM, Wood PJ. Use of congo red-polysaccharides interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl Environ Microbiol 1982;4:777–80.