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Original Article

# Utilization of horticultural waste (Apple Pomace) for multiple carbohydrase production from *Rhizopus delemar* $F_2$ under solid state fermentation

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ABSTRACT

The brown rot fungus *Rhizopus delemar* $F_2$ 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 cellulose, 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.



## 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 Oxedoreductase); 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 extracellular 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

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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* $F_2$ 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 $F_2$

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 mgm$L^{-1}$) 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 20,000*g*. After centrifugation supernatant was applied to QlAshedder Mni spin column and centrifuged for 2 min at 20,000*g*. Flow though 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 6000*g*. flow though 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 ≥6000*g*. Flow though 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 20,000*g* 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 6000*g* and flow though so obtained was discarded to elute the DNA. PCR amplification was done to confirm the identity of the fungal strain $F_2$, the small subunit 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) Prawanoo 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 $Na_2HPO_4$, 3.0 g $KH_2PO_4$, 0.5 g NaCl, 1.0 g $NH_2Cl$ and separately sterilized solution of 1 M $MgSO_4$ (2 mL) and 1 M $CaCl_2$ (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].

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