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Short communication

# Rational approach for mutant selection of *Talaromyces verruculosus* IIPC 324 secreting biofuel cellulases—Assessing saccharification potential

Lavika Jain[a,b], Deepti Agrawal[a,b,*]

[a] Biotechnology Conversion Area, Biofuels Division, CSIR- Indian Institute of Petroleum, Mohkampur, Dehradun, 248005, India
[b] Academy of Scientific and Innovative Research (AcSIR), Chennai, Tamil Nadu, 600113, India

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ABSTRACT

The present study describes a rational approach for mutant selection of *Talaromyces verruculosus* IIPC 324, where its 6–8 h old germinated spores were exposed to UV irradiation at 254 nm for 1 h. In total 10 mutants were obtained with a kill rate of 99.99%. Based on cellulases assays, none of the mutants showed simultaneous enhancement with respect to all the three components namely cellobiohydrolase, endoglucanase and cellobiase. Assessing saccharification potential with sugarcane bagasse as the feedstock resulted in the selection of mutant UV-8. The 10th generation of this mutant registered endoglucanase activity and cellobiase activity of 381.91 ± 5.4 and 50.73 ± 0.5 IU/g, respectively, on 4th day of fermentation. Its enzyme cocktail released 12.97 ± 0.65 g L−1 of reducing sugar from dilute sulfuric acid pretreated sugarcane bagasse after 72 h at 60 °C. Thus random mutagenesis by UV irradiation resulted in obtaining a stable mutant, whose endoglucanase productivity and hydrolytic potential, both improved by ~1.48 and ~1.4 fold, respectively, when compared to its parent strain.

## 1. Introduction

Rapid usage of fossil fuels and depletion in their reserves had triggered nations to expand their research horizons, tapping potential renewable resources for their energy security. These renewable sources of energy include wind, sun, water and biomass. Among the various renewable resources, biomass represents one of the most studied and rewarding options that can provide sustainable supply chain of versatile products such as biofuels, bio-based petrochemicals and bio-power (Somerville, 2006; Fernando et al., 2006).

Second generation biomass more popularly known as lignocellulosic feedstock specially has gained considerable importance due to its abundant and sustainable supply, inexpensive and renewable nature, low greenhouse gas emissions, no controversy on food *vs*. fuel, and efficient land usage. Considering Indian scenario, in year 2011–12 there were 527 working sugar mills that crushed around 240 million tonnes of cane per year. It generated 80 million tonne of wet bagasse (50% moisture), of which ~70 million tonnes was consumed as captive fuel for meeting the requirements of power and steam (Dhampur Sugar Mills Limited, 2012). Generally, sugarcane bagasse (SCB) is composed of 35–45% cellulose, 26–36% hemicellulose and 11–25% lignin (Chandel et al., 2012). Being a homo-polymer of glucose, cellulosic fraction has tremendous potential and can be a sustainable source of fuels and chemicals. However, these industries are highly reluctant to produce valorised products from sugarcane bagasse (SCB), hindering in the indicative target of Indian government for 20% ethanol blending produced from non-food feedstocks (MNRE, Government of India, 2009).

The recent valuation of global biofuel market ($168.18 billion; Year 2016) and its projected compound annual growth rate (CAGR) of 4.92% highlights the importance and demand of second generation biofuels (Biofuels International, 2016). The biotechnological route of this segment is primarily driven by enzymes mainly cellulase, which selectively depolymerise cellulose, an imperishable source of fermentable sugars.

Even today, the most advanced formulation of Novozymes and Dupont have used mutations and genetic interventions as tools along with bioprocess development to enhance cellulase production in *Trichoderma reesei* (Bischof et al., 2016). Similarly, China had been producing industrial cellulases from *Penicillium oxalicum* mutant (JU-A10-T) since 1996 which is catering diverse industries including food, feed, textile and biofuels (Liu et al., 2013). Notably, this mutant was also obtained by a series of physical and chemical mutagenesis along with adaptation strategy. Thus industrial success, economics, ease to carry out classical strain improvement (CSI) and no prior requirement of detailed knowledge of microbial genome had prompted many researchers to increase cellulase productivity in a number of filamentous fungi. Enhanced cellulase production using single or combinations of

* Corresponding author at: Biotechnology Conversion Area, Biofuels Division, CSIR- Indian Institute of Petroleum, Mohkampur, Dehradun, 248005, India.
*E-mail address:* deepti@iip.res.in (D. Agrawal).

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various mutagens such as UV irradiation, microwave, gamma radiations, Ethyl methanesulfonate (EMS), *N-Methyl-N'-nitro-N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) had been attempted in *Penicillium*, *Trichoderma*, *Aspergillus*, *Rasamsonia* etc (Solov'eva et al., 2005; Li et al., 2010; Vu et al., 2011; Pel, 2015).

Earlier, Jain and Agrawal (2018) have reported the isolation of *Talaromyces verruculosus* IIPC 324 from wood decaying soil and termite rich zones of CSIR-IIP (India) that produced highly specific and balanced cellulase cocktail for efficient hydrolysis of SCB. Therefore the main aim of the present study was to enhance cellulase productivity of *T. verruculosus* IIPC 324, using UV mutagenesis and selection of best mutant using a rational approach. It involved assessing the efficacy of the cellulase cocktails for effective deconstruction of SCB, a potential industrial waste from the sugar industry. Further validation trials were conducted with the 5th and 10th generation of promising mutant to check the stability of the said mutant and also confirm the data reproducibility, as observed during the preliminary screening.

## 2. Materials and methods

### 2.1. Raw materials and chemicals

All chemicals and media components were either procured from Sigma Aldrich, USA or Hi-Media Laboratories (Mumbai, India) and were of analytical or laboratory grade. Wheat bran was used as the substrate for solid state fermentation (SSF) and was purchased from local market. Raw SCB was procured from Doiwala Sugar Mill (Dehradun, India). Acid pretreatment was conducted by exposing raw SCB to 1.25% (v/v) dilute sulfuric acid and steam, solid: liquid ratio being 1:8, at a temperature of 140 °C with a holding time of 90 min (Ghosh et al., 2015). The biomass obtained after acid pretreatment was used for enzymatic saccharification studies. The said biomass was designated as acid pretreated SCB.

### 2.2. Microorganism

*Talaromyces verruculosus* IIPC 324 used in the present work was isolated and purified from wood decaying and termite rich zones of CSIR-IIP, India as described earlier (Jain and Agrawal, 2018). The fungal isolate was routinely maintained on potato dextrose agar (PDA) slants at 4 °C.

### 2.3. Mutagenesis, mutant selection and their stability

*Talaromyces verruculosus* IIPC 324 was grown on PDA for 4 days at 30 °C. Spores from the plates were transferred into10 ml potato dextrose broth (PDB) for germination at 37 °C for 6–8 h at 160 rpm. The germinated spores were counted using Neubauer's Chamber with the count being $5.5 \times 10^6$ spores/ml. Two ml of these germinated spores were exposed to short UV irradiation (254 nm) in dark chamber, 10 cm apart from the lamp for one hour. After this treatment, the exposed spores were spread on selective medium (Mandel's medium supplemented with 0.5% Sodium salt of carboxymethyl cellulose and 0.5% Avicel PH-101). The kill rate (%) during mutagenesis was calculated by the formula:

$$\text{KillRate}(\%) = \frac{(\text{Numberofgerminatedsporesexposed} - \text{Numberofmutantcolonies})}{(\text{NumberofgerminatedsporesexposedtoUVirradiation})} * 100$$

All the procedures up to plating and incubation were carried out in dark conditions to avoid DNA repair via photoreactivation (Friedberg, 2008).

Obtained mutants were sub-cultured and preserved on PDA until use. Later, all the mutants were grown in PDB which served as inoculum for solid-state fermentation conditions. The evaluation was carried out for all mutants with respect to cellulase production and enzymatic saccharification.

Repeated sub-culturing was carried out on PDA plates for mutant stability studies. Cellulase production and enzymatic saccharification studies were conducted up to 10th generation with the most promising mutant. This activity validated the reproducibility of the mutated strain in terms of cellulase production, consistent hydrolytic potential and its stability as well.

### 2.4. Solid State Fermentation (SSF)

Five gram of substrate was weighed in 100 ml Erlenmeyer flasks and moistened with 2.5 ml distilled water. The flasks were sterilized by autoclaving at 121 °C for 15 min at 15 lbs pressure. Flasks were inoculated with 6 ml of 24 h old mycelia (~10–15 mg dry mycelia wt/ml) of various mutants and their parent (*Talaromyces verruculosus* IIPC 324) individually. 0.5 ml of sterile 16.2% ammonium chloride was added as an additional nitrogen source for enhanced cellulase production (optimization data not shown). The flasks were incubated at 24 °C with initial moisture content being 62.5 ± 0.5% (optimization data not shown). After 4 days of the incubation period, the flasks were withdrawn, subjected to drying at 45 °C, bottled and stored until use. All the experiments were performed in triplicates, and the data was calculated as an average ± standard deviation.

### 2.5. Enzyme Extraction and Cellulase assays

Enzyme extraction was carried out by thoroughly mixing 0.5 g of dry mouldy bran with 50 ml distilled water for 2 h, followed by filtration and centrifugation (7500 rpm; 10 min; 4 °C). The filtrate served as crude enzyme extract (CEE) in which cellulase assays were conducted. Since a typical cellulase mixture for biofuel application requires synergism between all the three components, all the three assays namely endoglucanase (CMC'ase), cellobiohydrolase (CBH) and cellobiase (CBU) were performed during mutant screening studies.

Endoglucanase assay was carried out incubating 1 ml of appropriately diluted enzyme extract with 1 ml of 1% sodium salt of carboxymethyl cellulose (Sodium salt of CMC; Fluka- 21902) prepared in 50 mM citrate buffer (pH-4.0) at 60 °C for 10 min. The reaction was arrested by adding 3 ml of 3, 5, Dinitrosalicylic acid (DNS) reagent followed by boiling for 5 min for colour development. Reducing sugars were determined spectrophotometrically (U-2900 Hitachi Make) at 540 nm with glucose as standard (Miller, 1959). One unit of endoglucanase activity was defined as the amount of enzyme which released 1 µmole of glucose/min under the conditions indicated. Cellobiase assay (CBU) was carried out as per IUPAC protocol described earlier (Ghosh, 1987). One unit of cellobiase activity was defined as the amount of enzyme which released 2 µmoles of glucose/min under the optimized conditions. CBH assay was carried out at 60 °C by incubating 100 µL of a suitably diluted enzyme with 200 µL of 50 mM p-nitrophenyl β-D *p*-nitrophenyl β-D-cellobioside (pNPC) as a substrate in 50 mM citrate buffer (pH-4.0) in a total volume of 1 ml. After 20 minutes of incubation, the reaction was stopped by adding 1 ml of 2 M sodium carbonate solution. The absorbance of 4-nitrophenol liberated was measured at 405 nm spectrophotometrically. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmole of *p*-nitrophenol/min from pNPC under optimized assay conditions carbonate (Despande et al., 1984). All the enzyme activities were reported in terms of International Units (IU) per gram dry mouldy bran.

### 2.6. Enzymatic Saccharification Studies

Enzymatic saccharification of acid pretreated sugarcane bagasse was chosen as final selection criterion during mutant screening studies