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Solid state fermentative lignocellulolytic enzymes production, characterization and its application in the saccharification of rice waste biomass for ethanol production: An integrated biotechnological approach

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ABSTRACT

This work evaluates lignocellulolytic enzymes production by a selected strain *Streptomyces* sp. MDS cultivated in various agricultural wastes under solid-state fermentation (SSF). Maximum activities (U/gds) of endoglucanase (132.6 ± 1.15), exoglucanase (14.6 ± 0.62), cellobiase (125.6 ± 1.75), filter paperase (19.7 ± 0.42), amylase (278.5 ± 3.53), and xylanase (342.5 ± 3.36) were produced with rice waste biomass (RWB). Operational parameters and supplementation of nitrogen sources, metal additives, and surfactants were systematically optimized with a view to maximizing enzyme production. The harvested enzyme exhibited good stability at high pH (5 to 8) and temperature (50 to 80 °C) and showed robust nature in the presence of organic solvents, surfactants, and commercial detergents. The potential of crude enzyme mixture for hydrolysis of mild alkaline pretreated RWB was demonstrated, resulting in equivalent saccharification (64.85 ± 1.11%) relative to commercial enzymes (69.41 ± 1.15%). Yeast co-culture of *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* resulted in higher ethanol production (21.12 ± 0.82 g/L) and sugar consumption (88%) from enzymatic RWB hydrolysates (50 g/L) than monoculture. Finally, the leftover spent slurry from SSF effectively decolorized individual dyes and the actual textile wastewater, which increases the practical applicability of the developed bioprocess.

1. Introduction

Increasing agricultural operations produce huge amounts of biomass waste, the annual global primary production of biomass is about 220 billion tones on dry weight basis, which can be used as a starting material for new value-added products and help to solve environmental problems associated with its disposal [1,2]. However, the development of biotechnological applications of lignocellulosic biomass requires an understanding of its biodegradation. Its recalcitrant nature, crystallinity, and low fiber porosity as well the presence of lignin make lignocellulosic biomass difficult to hydrolyze [1]. Major improvements in the efficiency of lignocellulosic biomass hydrolysis can be achieved with hydrolytic enzymes, including cellulases as well as hemicellulases [3–5]. Recently, there has been an increasing trend toward producing several enzymes by means of solid-state fermentation (SSF) where fermented product can be directly used as an enzyme source [5,6]. Utilization of agro industrial residues considered the best substrates by virtue of their low cost, and huge availability [1,6,7]. Currently, enzyme technology focuses on studies that attempt to enhance enzyme activity and stability under non-conventional conditions that are more suited to industrial application and could be used for the pretreatment of biomass [8,9].

Biological ethanol production from lignocellulosic biomass appears to be a very attractive, cost-effective, and sustainable

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alternative energy source for the foreseeable future [10]. However, major problems with this process are the conversion of lignocellulosics into fermentable sugars, assimilation of mixed (6C and 5C) sugars derived from biomass, and their conversion into ethanol by microorganisms [10,11]. *S. cerevisiae*, which is traditionally used for ethanol production, can efficiently convert 6C glucose into ethanol but lacks the ability to utilize 5C xylose [11,12]. *K. marxianus* is generally recognized as a potential ethanol producer by virtue of its higher growth and ethanol fermentation efficiency at higher temperatures (37-45 °C), and ability to metabolize a variety of economically relevant industrial substrates [13]. Increasing the ethanol yield requires the utilization of pentose sugars (D-xylose) resulting from hydrolysis. By co-culturing *S. cerevisiae* and *K. marxianus* at an appropriate ratio, it may be possible to obtain a higher ethanol yield by utilizing mixed sugar from lignocellulosic biomass hydrolysates. This approach was investigated in this study.

Industrial effluents containing textile dyes pollute water bodies, affect aquatic ecosystems, and have a serious impact on human health owing to their toxic and carcinogenic properties. Thus need to be decontaminated before their disposal [14]. Of the different conventional physicochemical methods, microbial system including; bacteria, fungi, algae and plants or enzymatic (using oxidative and reductive enzymes) decolorization and degradation is an ecofriendly, cost-competitive alternative to physicochemical treatment methods [14]. Moreover adsorption may be seen as suitable because of its low cost, easy to operate, and effectiveness in binding with toxic substances. Adsorption on low-cost adsorbents makes removal of dyes from wastewaters ecofriendly and cost effective [15]. We have therefore explored the additional utilization of the resultant solid waste after enzyme extraction for the treatment of dye wastewater.

In this study, we have selected our reported potential isolated strain *Streptomyces* sp. MDS [3,16] for lignocellulolytic enzyme production under SSF by utilizing various agricultural waste biomasses. Effects of different operational conditions and nutritional parameters to maximum enzyme secretion and enzyme characterization were determined. The application of crude enzyme mixture for the hydrolysis of RWB followed by ethanol production by mono- and co-cultures of yeast was studied. The resulting spent fermented slurry was studied for treating individual dyes and actual textile wastewater. The integrated biotechnological process seems to be a novel and useful for the efficient utilization of agro-industrial waste and by-products.

2. Materials and methods

2.1. Lignocellulosic biomass and pretreatment conditions

Lignocellulosic raw materials such as, rice waste biomass, wood straw, local grass powder, sugar cane barboja and sugar cane bagasse were chosen as the carbon substrates for this study. The raw materials were air dried, milled, and sieved through 0.2 mm, 0.5 mm, and 1.0 mm screens and stored at room temperature. Pretreatment of RWB (particle size, 0.5 mm) was carried out separately under mild alkaline (1% (w/v) NaOH) conditions, and it was autoclaved at 121 °C for 15 min. Pretreated samples were washed to neutral pH and dried at 60 °C until the weight no longer changed then stored in airtight containers at 4 °C.

2.2. Microorganism and culture conditions

The selected strain, *Streptomyces* sp. MDS, was isolated, and the detailed results of 16S rRNA sequencing and phylogenetic analysis have been reported elsewhere [2]. The culture was maintained on Dubos salt medium (g/L): $NaNO_3$, 0.5; K_2HPO_4, 1.0; $MgSO_4{\cdot}7H_2O$, 0.5; KCl, 0.5; $FeSO_4{\cdot}7H_2O$, 0.01; CMC, 10; agar powder, 20; at pH 6.5. It was stored at 4 °C and subcultured monthly.

2.3. Lignocellulolytic enzyme production under solid-state fermentation (SSF)

The production of cellulose, hemicellulose and lignin degrading enzymes in the presence of different agricultural waste biomasses by *Streptomyces* sp. under solid-state fermentation was determined. SSF was carried out in 250 mL Erlenmeyer flasks, each containing 5.0 g of dry lignocellulosic biomass, which had been moistened with Dubos salt medium to attain the final substrate-to-moisture ratio and sterilization was carried out by autoclaving (121 °C for 15 min). 1 mL of spore suspension (absorbance of 1.0 at 600 nm) of *Streptomyces* sp. prepared in sterile saline from 8-day-old culture was used for inoculation. Enzyme production and its extraction followed the procedures previously reported [4]. The effects of incubation time (1–10 days), incubation temperature (25–40 °C), initial pH of the medium (4–7), moisture content (1:1 to 1:2), and substrate particle size (0.2–1.0 mm) on enzyme production by *Streptomyces* sp. from RWB under SSF were investigated. The possibility of enhancing enzyme production by supplementing with various nitrogen sources (0.2% w/w each), metal additives (0.2% w/w each), and commercial surfactants (0.1% v/v each) was evaluated.

2.4. Enzyme assays

Endoglucanase, exoglucanase, xylanase and amylase activities were determined by following the methods previously reported [3,5]. Endoglucanase activity was assayed using a reaction mixture containing enzyme solution (1 mL) with 1% carboxymethyl cellulose (CMC) (1 mL) in McIlvaine's buffer (0.1 mol/L citric acid-0.2 mol/L phosphate buffer; pH 5), further incubated at 50 °C for 30 min. Exoglucanase assay was performed using a reaction mixture containing enzyme solution (2 mL) with 1 mL of 1% avicel in McIlvaine's buffer and incubated at 50 °C for 2 h. For xylanase and amylase enzyme activity were measured in a reaction mixture consisting of 1 mL of enzyme solution diluted appropriately in McIlvaine's buffer with 1 mL of aqueous suspension of 1% xylan or 1% starch at 50 °C for 10 min. The reaction mixture of above assays was terminated by the addition of 2 mL of dinitrosalicylic acid reagent and further heating in boiling water bath for 10 min. One unit of enzyme activity in each case was defined by the amount of enzyme that produces one micromole of reducing sugar (glucose or xylose) from the substrate per minute. Filter paperase activity was assayed in a reaction mixture containing a string of filter paper (Whatman No. 1; 1.0×6.0 cm $\approx$ 50.0 mg), 0.5 mL of 50 mM citrate buffer (pH 5.0), and 0.5 mL of diluted enzyme solution was incubated at 50 °C for 60 min [7]. Cellobiase activity was determined by assaying the release of *p*-nitrophenol from *p*-nitrophenyl-β-glucopyranoside (pNPG) [12]. One unit of cellobiase activity was defined as the amount of enzyme required to release 1 μmol of *p*NP per minute under standard assay conditions. Lignin degrading enzymes such as lignin peroxidase, Mn-peroxidase, and laccase were assayed spectrophotometrically by following the procedure reported earlier [17]. One unit of enzyme activity was defined as the change in absorbance unit per minute per mg of protein of the enzyme. All enzyme assays were performed in triplicate and the average rates were calculated to represent the enzyme activity.

2.5. Enzyme characterization studies

Enzymes harvested from *Streptomyces* sp. after 8 days of incubation with RWB were used to determine enzyme stability studies. Enzyme stability was investigated after incubation of the enzymes for 1 h at different temperatures (50–90 °C) but constant pH (5.0),

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