

Ragi Husk as Substrate for Cellulase Production Under Temperature Mediated Solid State Fermentation by *Streptomyces* Sp

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Abstract: Cellulases have diversity of industrial applications and their cost effective production using agroindustrial wastes by solid state fermentation poses an efficient method. Actinomycetes are considered highly valuable due to their secondary metabolites production and in this study, an attempt was made to optimize the use of ragi husk and refine the process of cellulase production by temperature mediated solid state fermentation. Actinomycetes were isolated from paper mill industry soil and cellulase producing *Streptomyces* sp was selected for the experiments. Ragi husk was used as substrate for solid state fermentation of cellulase and varying incubation temperatures (20°C, 25°C, 30°C, 35°C and 40°C) was considered to determine its effect on enzyme activity after 6th, 9th and 12th day of fermentation. The carboxymethyl cellulase (CMC-ase) activity was measured and the observations obtained were compared with the standard glucose curve to determine the amount of reducing sugar ($\mu\text{g ml}^{-1}$) released. Enzyme activity was highest at 35°C and was recorded as 35.14, 45.90 and 59.56 IU ml^{-1} at the end of 6th, 9th and 12th day of fermentation. Highest amount of reducing sugars at a concentration of 322 $\mu\text{g ml}^{-1}$ was released at the end of 12th day at 35°C. The results indicated that the enzyme activity was temperature dependent while using ragi husk as growth substrate under solid state fermentation.

Keywords: Cellulase, *Streptomyces*, Solid State Fermentation, Agroindustrial Wastes, Ragi Husk, Actinomycetes

1. Introduction

Cellulose, a linear polymer of glucose molecules joined by β -1,4-glycosidic-linkages, is the most abundant biopolymer on the planet and strongly resistant to biodegradation [1]. Cellulose is commonly degraded by cellulases (β -1,4 glucan hydrolases) that hydrolyze cellulose and produce as primary products glucose, cellobiose and cello-oligosaccharides. They are a group of enzymes which act synergistically on cellulose to bring about its hydrolysis and are currently the subject of numerous studies due to their importance in biomass hydrolysis.

Cellulases have attracted much interest because of the diversity of their application. The major industrial applications of cellulases are in textile industry for biopolishing of fabrics and producing stonewashed look of denims, as well as in household laundry detergents for

improving fabric softness, brightness and anti-deposition. Besides, they are used in animal feeds [2] for improving the nutritional quality and digestibility, in processing of fruit juices and in baking, while de-inking of paper is yet another emerging application. Because of its commercial importance and complexity of structure, research of many labs and industries are revolving around cellulase producing microbes. Cellulases are produced by fungi [3-6], bacteria [7, 8] and actinomycetes [9-11]. Though several microorganisms produce cellulases, relatively few numbers of fungi and bacteria are known to produce the enzymes in high levels. Actinomycetes are Gram positive, filamentous, spore forming, aerobic bacteria. Actinomycetes are considered highly valuable as they produce various secondary metabolites and other biologically useful compounds such as antibiotics, vitamins, nutritional materials, herbicides, pesticides and enzymes [12-14]. They are able to metabolize

various compounds of complex structures by the production of extracellular enzymes [15].

Millets are cultivated in a wide range of soils and climates because of their hardy nature and short growing seasons making them important crops in semi-arid regions, worldwide. Millet bran, by-product of milling process is an ideal, cheaper source and mainly used as animal feed due to their high nutritive value and low costs. Finger millet (*Eleusine coracana*) locally known as Ragi is predominantly starchy and improve the C/N ratio, presenting ideal conditions for growth of microbes. Efforts have been made to obtain a low cost cellulase, using different technologies and substrates. In this context, ragi husk appear as ideal substrate for microbial fermentation, due to their rich content of organic components, low cost and wide availability. Furthermore, the use of agricultural by-products presents a better energy balance and a lower environmental impact than those of pure substrates [16].

Solid state fermentation (SSF) has been described as the process that takes place in a solid matrix (inert support or support/substrate) in the absence or near absence of free water [17], but the substrate requires moisture to support the growth and metabolic activity of microorganisms [18]. Solid state fermentation is a suitable strategy for producing low cost enzymatic products. Nutrients present in the substrate support growth and due to the natural metabolism of the microorganism, can secrete enzymes, while growing in the solid substrate [19]. Here, an attempt was made to optimize the use of ragi husk and refine the process of cellulase using actinomycetes, so as to enhance the end product output that is to maximize the yield of cellulase by temperature mediated solid state fermentation.

2. Materials and Methods

2.1. Isolation of Actinomycetes

Soil samples were collected from paper mill industry located at outskirts of Bangalore. Starch-casein-agar was used to isolate actinomycetes colonies from the soil and the media was supplemented with rifampicin and cycloheximide at $50 \mu\text{g ml}^{-1}$ concentration. To isolate actinomycetes, 5 g of screened soil (1 mm sieve) were added to 50 ml sterile water and shaken at 140 rpm for 20 min. Ten fold dilutions were prepared from the resulting suspension and was serially diluted up to 10^{-10} with sterile distilled water. One ml of each dilution was spread on the surface of starch casein agar plate containing 20 ml of each medium. After 7 days of incubation, the colonies showing the characteristics of actinomycetes were observed. The pure culture was maintained as a spore suspension in 20% glycerol at -20°C .

2.2. Primary Screening of Isolates for Production of Cellulase

CMC Agar (carboxymethylcellulose 0.5 gL^{-1} , NaNO_3 0.1 gL^{-1} , K_2HPO_4 0.1 gL^{-1} , MgSO_4 0.05 gL^{-1} , yeast extract 0.05 gL^{-1} , Agar 15 gL^{-1}) was used for cellulose degrading

efficiency test [2]. The selection of cellulase producers was based on the extent of production of clear zones on CMC agar when treated with 1% Congo red dye (w/v) followed by 1 N HCl for 15-20 min.

2.3. Characterization of Cellulase Producing Actinomycete

The actinomycete colony selected from primary screening was identified based on cultural and morphological characteristics according to standard protocols of International *Streptomyces* Project [20]. The cultural characteristics such as colour of substrate and aerial mycelia, growth pattern, colony texture, and colony reverse colour were examined [21].

2.4. Inoculum and Substrate Preparation

A loopful of actinomycetes culture was transferred to 10 ml of autoclaved water and used as inoculum. 40 g of ragi husk was taken in a 250 ml flask and 40 ml of minimal salt media [$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ -1.1g, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ -0.61g, KCl-0.3g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -0.01g, pH-7, Distilled water-100 ml] was added (1:1 w/v). The moisture content was maintained at optimum levels and the contents of the flask were autoclaved at 121°C for 20 mins.

2.5. Solid State Fermentation

After autoclaving, the contents of the flask was inoculated with 1.0×10^7 cfu/ml of 7 days old culture of *Streptomyces* sp and incubated under static conditions. Varying incubation temperatures (20°C , 25°C , 30°C , 35°C and 40°C) was considered to determine its effect on enzyme activity after 6th, 9th and 12th day of fermentation from 40 g of substrate used. Each experiment was done in triplicates. At the end of fermentation period, 50 ml of citrate buffer (pH 4.8) was added to the cultured flasks followed by filtration through 0.5 mm nylon cheese cloth. The filtrate was centrifuged at 10000 rpm for 5 mins and the cell-free culture supernatant was used as the source of enzyme.

2.6. Enzyme Assay

Carboxymethyl cellulase (CMC-ase) activity was assayed using the method described earlier [22]. 0.5 ml of enzyme solution was added to 0.5 ml of 0.55% (w/v) of carboxy methylcellulose prepared in sodium citrate buffer (pH 4.8) and incubated at 37°C for 60 min. The reaction was stopped by adding 1.0 ml of 3,5-dinitro salicylic acid (DNSA) reagent to the mixture followed by boiling for 10 min and adding 8.0 ml of distilled water. The Optical Density of the mixture was recorded at 540 nm using a UV-Vis spectrophotometer and compared with the standard glucose curve to determine the amount of reducing sugar ($\mu\text{g ml}^{-1}$) produced during cellulose hydrolysis.

2.7. Reducing Sugars Content Assay

The cellulase activity of actinomycete was measured by determining the amount of reducing sugars liberated by using

a di-nitro salicylic acid (DNSA) colorimetric method [23]. Reducing sugars analysis was conducted by using 2 ml of sample which was added to 3 ml of DNS and boiled for 15 min. After cooling, 1 ml of Rochelle salt solution was added followed by measuring the absorbance at 575 nm using a spectrophotometer against the blank of distilled water.

3. Results and Discussion

Actinomycetes colony obtained in starch casein nitrate agar was purified by streaking (Figure 1) and the colony that has produced clear zone around in CMC agar (Figure 2) upon addition of congo red was further identified by cultural and morphological characteristics. The results revealed that the colony belongs to *Streptomyces* sp which was further used for cellulase production. Ragi husk was used as substrate to produce cellulase enzyme by solid state fermentation under varying temperature. The enzyme was recovered from the fermentation media by adding citrate buffer (pH 4.8) and the crude enzyme was used for enzyme assay.



Figure 1. Actinomycete isolate from paper mill industry soil.

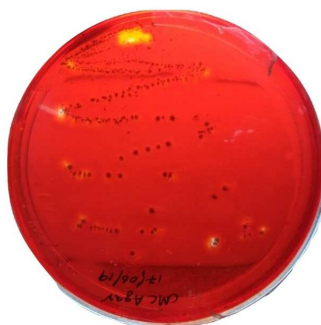


Figure 2. CMC-ase activity (zone of hydrolysis) of the isolate upon addition of congo red solution.

Environmental variables significantly influence microbial growth and product formation in SSF. This work sought to address the optimum temperature for the production of cellulase activity recorded at different temperatures. Optimal temperature for cellulase enzyme production by *Streptomyces* was recorded at temperatures 25°C, 30°C, 35°C, 40°C and 45°C on 6th, 9th and 12th day of incubation using ragi husk as growth substrate. The CMC-ase activity was measured and the observations obtained were compared with the standard glucose curve to determine the amount of reducing sugar ($\mu\text{g ml}^{-1}$) released. The result of investigation carried out in this study on effect of temperature on cellulase by *Streptomyces*

sp is depicted in Figure 3. Cellulase assay indicated that the enzyme activity was temperature dependent and the activity was increased when the fermentation temperature was increased through 25°C to 35°C. In all the time intervals tested (6th, 9th, 12th day), the enzyme activity was highest at 35°C and was in the range of 35.14-59.56 IU ml^{-1} . The activity was reduced when the temperature was increased from 35°C to 40°C, however, it started increasing again when the temperature was set as 45°C at the end of 6th day. The release of reducing sugars was determined by DNSA method and the results indicated that highest amount of reducing sugars at a concentration of 322 $\mu\text{g ml}^{-1}$ was released at the end of 12th day at 35°C. It was also noted that at the end of 6 days of fermentation, more amount of reducing sugars were (216 $\mu\text{g ml}^{-1}$) released at 45°C (Figure 4).

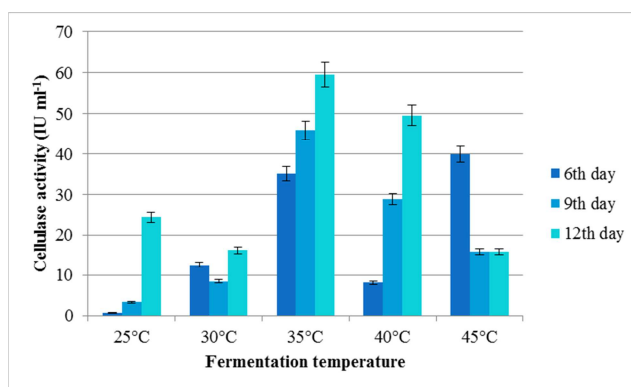


Figure 3. Cellulase activity of *Streptomyces* sp under varying fermentation temperature using ragi husk as growth substrate.

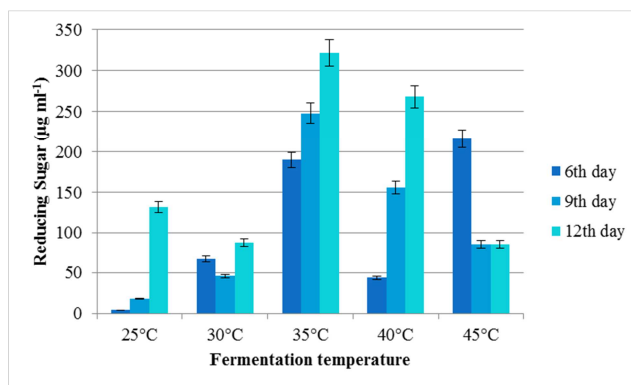


Figure 4. Reducing sugar release by *Streptomyces* sp under varying fermentation temperature using ragi husk as growth substrate.

The use of agro-industrial residues and by-products as feedstock enriches the SSF processes economic value. The substrates used in SSF make the process eco-friendly because it also solves the problem of their disposal, which otherwise would cause pollution. The genus *Streptomyces* is widely found in soils and it is well known for its production of many extracellular enzymes, including those involved in the degradation of cellulose, hemicellulose and lignin, the three primary plant cell-wall polymers [24-26]. *Streptomyces* sp. are also indicated for SSF processes since they present characteristics such as abundant colonization of solid

residues, production of a wide range of degradative enzymes and high resistance to extreme conditions [27].

Optimization of environmental variables and culture medium factors is a key concern for the development of suitable biotechnology for cellulase production [28]. Temperature significantly influenced the growth, development and, in general, metabolic activities of an organism. Hence, it was essential to optimize temperature for maximum cellulase production in *Streptomyces* under SSF, using ragi husk as substrate. In this study, temperature was found to influence extracellular enzyme secretion and 35°C was found optimum for cellulase production by *Streptomyces* sp. In a study by Prasad et al., [29], *Streptomyces griseorubens* showed highest cellulolytic activity at 45°C on 6th day of incubation period. In another study, *Streptomyces* sp. secreted on third day (0.19 ± 0.005 U/gds) of fermentation [30].

Incubation temperature is paramount for optimal enzyme production due to alterations in microbial protein structure and properties with temperature variations [31]. Previous studies have reported optimum temperatures of 30°C for cellulase production by two *Streptomyces* strains [32]. In addition, Jaradat et al., [33] also reported optimal temperature of 30°C for cellulase production by *Streptomyces* sp. strain J2. It was revealed from the study that although optimal cellulase production was recorded at temperature of 35°C with activity of 59.56 U ml⁻¹, production was also recorded at all the incubation temperatures tested. Other temperatures at which cellulase production was recorded in order of descent are: 45, 30, 25 and 40°C with activity at the end of 12 days. Fatokun et al., [34] reported that cellulase production was optimal at incubation temperature of 40°C and 0.26 U ml⁻¹ activity was attained at 60 h with specific productivity of 205 U g⁻¹.

4. Conclusion

Optimal condition for cellulase production by *Streptomyces* was investigated by measuring cellulase activity at the varied incubation temperature conditions at different time intervals. Enzyme production was favored by 35°C incubation temperature during the 12 days fermentation time. Furthermore, the strain was able to produce higher amounts of cellulase using ragi husk as growth substrate under solid state fermentation. In this work, both the cultivation and fermentation conditions were optimized to enhance the cellulase production by *Streptomyces* and the cellulolytic potential of the isolate could be beneficial in hydrolysis of the cellulosic materials.

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