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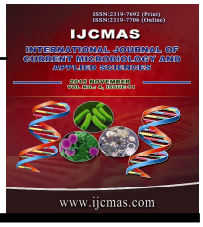

Original Research Article

Exploration of Agrowastes for the Production of Cellulase by *Streptomyces* DSK29 under Submerged and Solid State Systems

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Keywords

Actino bacterium, Agrowastes, Bioprocess, Cellulase

A B S T R A C T

A prominent cellulolytic actinobacterium was isolated from limestone quarry soil and was identified as *Streptomyces* DSK29. Cellulase production was carried out using potent strain of actinobacterium employing various regional agrowastes. The effect of basic bioprocess variables on enzyme production was studied. Maximum cellulase production obtained was 44 IU under solid state bioprocess using sorghum stover as substrate, indicating that, solid state is more efficient then submerged bioprocess. The optimal conditions for the cellulase production were found to be initial inoculum size ($1x10^8$ spores/ml), incubation temperature (45°C), Particle size (2mm) and moisture content (65%). Results reveal that there is a scope for further enhanced production with higher cellulase titres under solid state bioprocess.

Introduction

Actinobacteria are the microbial group richest for the production of variable secondary metabolites. Most of these bioactive molecules are the end products of complex multistep biosynthetic pathways. This uniqueness of pathways and products are specific for a strain rather than for a given species or larger taxonomic group (Wolfgang, 1994). *Streptomyces* is considered as one of the most important genus of the actinobacteria. They were studied in depth because of their capacity to produce diverse and industrially important bioactive molecules especially enzymes. Actinobacteria, one of the known cellulase producers, has attracted considerable research interest due to its potential applications in recovery of fermentable sugars from cellulose. Increasing demand, rising cost of fossil fuels and global climatic changes have shifted global efforts to utilize renewable resources for the production of alternative energy (Rahna and Ambili, 2011). Bioprocess is referred to biological conversion of complex substrates in to simple value added products by various microorganisms. It is mainly categorized as submerged and solid state based on the conditions and type of substrates used in the bioprocess. Till date the cellulase production has been widely studied in submerged bioprocesses, but relatively high cost of

enzyme production has hindered the industrial application of cellulose bioconversion (Pandey et al., 1999). Solid state bioprocess is an attractive bioprocess to produce cellulase economically due to its lower capital investment and lower operating expenses. Another approach to reduce the cost of cellulase production is the use of agrowastes materials as substrates rather than expensive pure cellulose (Robinson et al., 2001). Cellulase production is the most important step in the economical production of ethanol, single cell protein and other chemicals from renewable cellulosic materials (Jian and Yang, 2007). Solid state bioprocess can be of special interest in bioprocesses where crude fermented product could be used directly as enzyme source. This is partly because bioprocesses involving solid state fermentation have lower energy requirements, produce less wastewater and are environment friendly as they resolve the problem of solid wastes disposal (Pandey, 2003).

Currently, industrial demand for cellulases is being met by production methods using submerged fermentation bioprocesses, employing genetic modified strains of *Trichoderma*. The cost of production in Submerged fermentation is however high and it is uneconomical to use them in many of the bioprocesses such as enzyme production which necessitates reduction in the production cost by deploying alternative methods such as solid state bioprocess (Harinder et al., 2008). In whole fermentation bioprocess, cost is one of the most important factors which affect the feasibility of the bioprocess at industrial scale. Main problem in cellulase production by fermentation is the utilization of expansive substrates (Irfan et al, 2012). In spite of the huge coverage of research for finding more active enzyme preparations from large variety of microorganisms, the enzymatic saccharification of lignocelluloses so far has not been reached to the level of conversion of starch into glucose by the microbial enzymes (Gomes et al., 2000). Thus much work and research is needed to produce enzymes capable of saccharifying the lignocelluloses employing solid state bioprocess.

Materials and Methods

1. Isolation and identification of actinobacterium

The culture of cellulolytic actinobacterium used in the present study was isolated on Starch casein agar (Kuster, 1961) from the soil of limestone quarry. It was identified based on standard colony characters, pigmentation pattern, microscopic features, biochemical and physiological properties (Goodfellow et al, 1988). The culture was maintained on starch casein agar and stored at 4°C.

2. Bioprocesses for the production of cellulase

The two major types of bioprocesses, namely submerged and solid state were evaluated for the maximum production of cellulase, employing *Streptomyces* DSK29 using regional agro wastes extracts. Rice bran, wheat bran, bengal gram husk, red gram husk, sorghum stover, bengal gram husk, corn husk, green gram husk and ground nut cake were major regional agrowastes collected from local market and evaluated as substrates for the production of cellulase. All agro wastes were cleaned in the laboratory by grinding at different particle sizes and sterilized by autoclaving at 121°C for 15 min.

Submerged bioprocess was evaluated as per the standard procedure (R). The extracts

from the agrowastes were obtained using ten gram of each of the powdered substrates by dissolving in 100 ml of distilled water in 250 ml Erlenmeyer's flask. The contents of the flask were heated for about 10 min and cooled to room temperature. It was filtered using Whatman filter paper no. 1. The extract thus obtained was used for submerged fermentation using mineral salt medium (Mandel et al., 1981), excluding starch. The pH of the medium was adjusted to 7.2 and autoclaved at 121°C for 15 min. The flasks were inoculated with one ml of spore suspension ($1x10^8$ spores/ml) of *Streptomyces* DSK29 and incubated at 35°C in shaker incubator at 180 rpm for 5 days. The cellulase activity was determined at every 24 h as per the standard procedure prescribed by Ghose (1987).

The solid state bioprocess was examined (Singhania et al., 2010) as follows: the solid substrate (10g) was weighed in 250 mL Erlenmeyer flasks and moistened with mineral salt broth (excluding starch) to obtain 65% of moisture content. The contents were sterilized and inoculated with 1ml inoculum ($1x10^8$ Spores/ml) of *Streptomyces* DSK29. The flasks were incubated for 5 days at 35°C. Samples were withdrawn at every 24 h using whole flasks for enzyme extraction by simple contact method (Singhania et al., 2006). Citrate buffer (0.05M, pH 4.8) was added to the fermented substrate to a total volume of 100 mL and mixed for 1 h on rotary shaker. The suspension was filtered and centrifuged and the supernatant was used as the crude enzyme preparation for assay of enzyme activity (Ghose, 1987).

Optimization of solid state bioprocess and assay of cellulase

Important bioprocess variables such, Inoculum size ($1x10^5$ to $1x10^9$ spores/ml, with an increment of $1x10^1$), Incubation temperature (30°C to 50°C, with an increment of 5°C), Particle size (2 to 10 mm, with an increment of mm), Moisture content (50% to 70%, with an increment of 5%) were assessed (Liu and Tzeng, 1998) employing solid state fermentation for their effect on cellulase production.

Cellulase assay was assessed (Ghose, 1987) by incubating 1.5 ml assay mixture, containing 0.5 ml of crude enzyme extract and 0.5% specific substrate in citrate buffer (0.5 ml with pH- 4.8) for 30 min at 50°C. The reducing sugar formed after the incubation was estimated by Di nitro salicylic acid (DNS) method (Miller, 1959).

Results and Discussion

Characterization of the potential actinobacterium

The actinobacterial culture isolated from limestone quarry was an efficient cellulase producer. Based on morphological, biochemical and physiological characters (Table 1) it was identified as genus *Streptomyces* and was designated as *Streptomyces* DSK29.

Quite a few number of novel isolates of actinobacteria in general and Streptomyces in particular were reported (Dastager et al., 2007a, 2007b; Raziuddin et al., 2012; Shivaveerkumar et al., 2014; Zainab et al., 2014) earlier from different ecological habitats of the region.

It appears that the regional habitats are the gold mines for rich diversity of actinobacteria, which can be explored further for potential bioactive molecules. Recently, actinobacteria are the sources of attraction for the production of several enzymes with myriad applications.

Production of cellulase using agrowastes as substrates

Comparative studies between submerged and solid state bioprocess claim higher yields and other advantages for products made by solid state bioprocess**,** similar or higher yields than those obtained in the corresponding submerged cultures. Interest in solid state bioprocess has been increasing because of its important applications in producing enzymes, biopesticides, aroma compounds, biopharmaceuticals, organic acids and other bioactive compounds. Agro-industrial residues are processed using solid state bioprocess because it has lower energy requirement, produce lesser waste water and are environment friendly.

Various regional agrowastes substrates mainly, Rice bran, Wheat bran, Bengal gram husk, Red gram husk, Sorghum stover, Bengal gram husk, Corn husk, Green gram husk and Ground nut cake were screened for the production of cellulase. Of all these sorghum stover exhibited highest production of cellulase employing both submerged and solid state bioprocess.

Table 2 shows the comparative results of cellulase production by *Streptomyces* DSK29 under submerged fermentation. A varied range of cellulase production by actinobacteria has been reported employing RSM for optimization and agrowastes such as corn steep liquor (Cirigliano et al., 2013) and wheat bran (Lima et al., 2005) as substrates. Sorghum stover was proven to be the better substrate for the production of cellulase (34 IU) at 120 h of t fermentation.

Table.1 Morphological, biochemical and physiological characters of an actinobacterial isolate DSK29

Morphological characteristics									
Colony	Medium, irregular, smooth and dry								
Mycelium	Aerial mycelium- Green, Substrate mycelium- Dark green								
Pigmentation	No synthesis of diffusible pigment								
Microscopic	Gram positive, spiral and less branching mycelium								
Physiological characteristics									
Effect of pH on growth	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0
	-	-	+	+	++	++	+++	++	+
Effect of temperature on growth (^{o}C)	30		35		40		45		50
	No growth		Poor growth		Huge growth		Moderate growth		Poor growth
Biochemical characteristics									
Substrate hydrolysis & H_2S production	Casein		Cellulose		Gelatin		Starch		Production of H_2S
	Positive		Positive		Positive		Positive		Positive

Table.2 Screening of agrowastes for production of cellulase in mineral salt medium by *Streptomyces* DSK29 under submerged system

Agrowaste substrates	Enzyme activity (IU) at different fermentation period				
	24h	48h	72h	96h	120h
Rice bran	4	10	15	18	15
Wheat bran	8	12	22	29	25
Bengal gram husk	8	18	21	25	22
Red gram husk	7	12	19	24	20
Sorghum stover	10	18	28	34	31
Black gram husk	5	10	13	20	18
Corn husk	9	18	20	23	19
Ground nut cake	4	9	11	15	12

Table.3 Screening of agrowastes for production of cellulase by *Streptomyces* DSK29 under solid state system

Agrowaste substrates	Enzyme activity (IU) at different fermentation period				
	24h	48h	72h	96h	120h
Rice bran	8	12	18	24	20
Wheat bran	10	14	22	35	31
Bengal gram husk	4	10	17	25	22
Red gram husk	5	9	12	22	18
Sorghum stover	10	18	32	38	34
Black gram husk	7	12	20	32	29
Corn husk	6	15	22	30	27
Ground nut cake	6	9	10	18	12

Fig.1 Effect of particle size on production of cellulase by Streptomyces DSK29 under solid state system

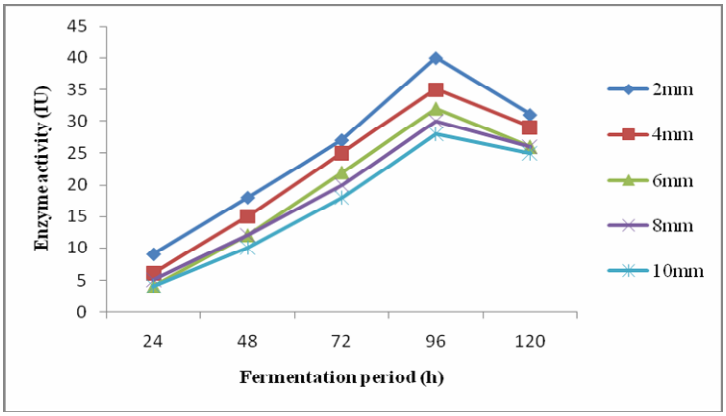


Fig.2 Effect of inoculum size on production of cellulase by *Streptomyces* DSK29 under solid state system

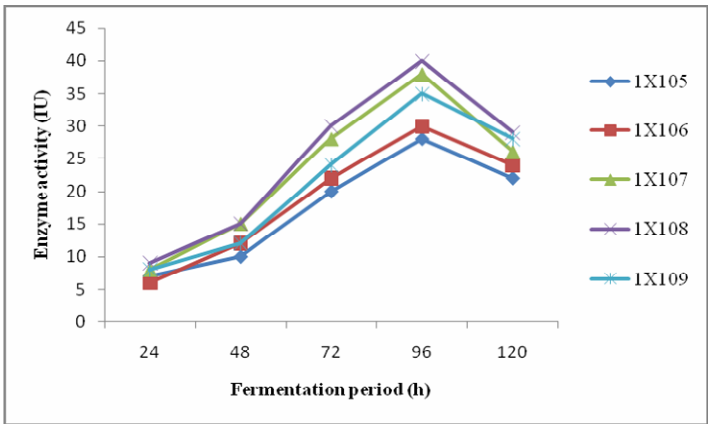


Fig.3 Effect of pH on production of cellulase by *Streptomyces* DSK29 under solid state system

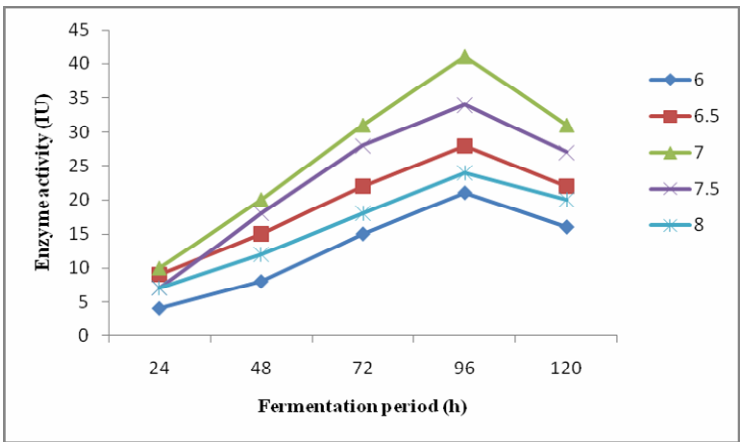


Fig.4 Effect of temperature on production of cellulase by *Streptomyces* DSK29 under solid state system

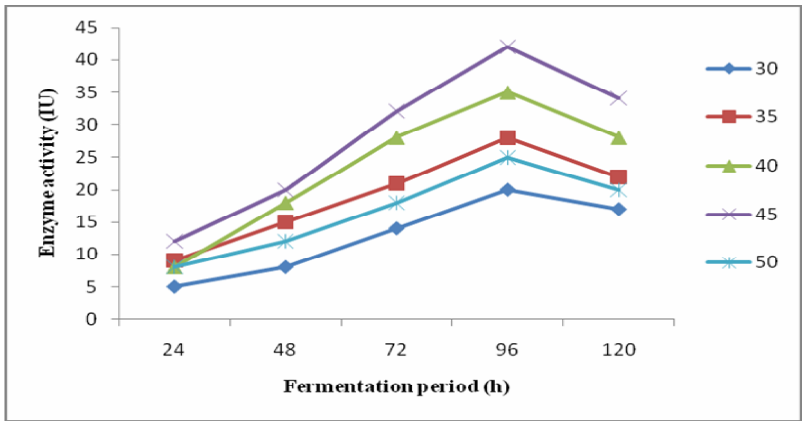
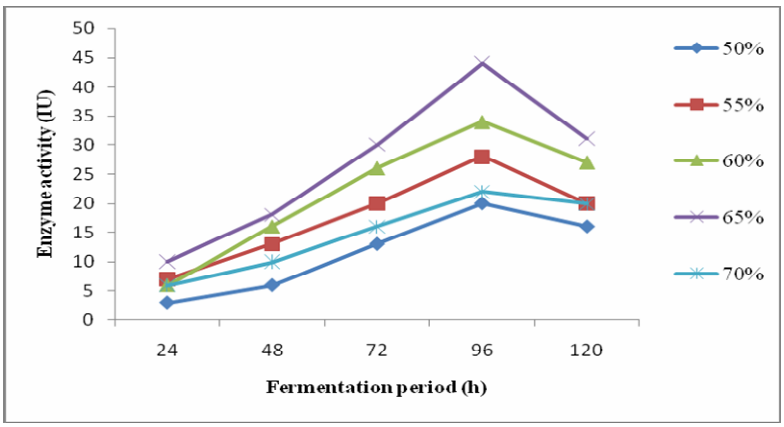


Fig.5 Effect of moisture content on production of cellulase by *Streptomyces* DSK29 under solid state system



Jian Liu and Jichu Yang (1998) reported cellulase production (23.76 IU) using lignocellulosic waste from vinegar industry employing solid state fermentation. Moses Jeyakumar Rajesh et al. (2012) reported maximum cellulase and β-glucosidase activity using *Trichoderma reesei* utilizing Rice bran and corn sraw as substrates. Table 3 indicates the production of cellulase under solid state bioprocess by *Streptomyces* DSK29. In the present investigation, sorghum stover was found to be better substrate for the production of cellulase under submerged (38 IU) and solid state (44 IU) bioprocess. All other agro waste substrates were not so encouraging for the production of cellulase.

Optimization of solid state bioprocess for the production of cellulase

During the process of optimization, identification of bioprocess variables for maximum production of cellulase is very important. Single factor studies indicated that, particle size of 2mm (Fig. 1), inoculum size of 1×10^{-8} spores/ml (Fig. 2), pH of 7 (Fig. 3), temperature of 45°C (Fig. 4), moisture content of 65% (Fig. 5) were recorded as optimum for the maximum production (44 IU) of cellulase under solid state bioprocess. Moses Jeyakumar Rajesh et al. (2012) reported optimization of solid state bioprocess yielded maximum production (18.5 IU) of cellulase. The present study reveals much higher production (44 IU) of cellulase compared to available reports.

In conclusion, Actinobacterium strain DSK29 isolated from limestone quarry is indigenous isolate having significant cellulolytic ability. Solid state bioprocess was found to be an effective system for the maximum production (44 IU) of cellulase using sorghum stover as substrate. *Streptomyces* DSK29 could be explored further for greater enhanced production and application of cellulase.

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