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Optimization of Cultural Conditions for the Production of Cellulase from Agrowaste Using *Penicillium brocae* PSKV2 under Solid State Fermentation

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A B S T R A C T

Recent interest by researchers to produce cellulase from cheaper substrate is justifiable. Recently, agro residues have been used as carbon sources in cellulase production in industrial biotechnology. To investigate the production and optimization of cultural conditions for production of cellulase under solid state fermentation (SSF) from *Penicillium brocae* PSKV2 using surface-modified jack fruit waste and corncobs as substrate. The soil samples from the bottom of decaying agrowaste dumps from different locations of Bengaluru rural area for the isolation of fungal strain. The fungal isolates were screened for cellulase production. Major regional agro wastes such as Jack fruit waste, corn cobs, paddy straw, sugarcane bagasse were screened for production of cellulase. The cultural condition for optimum production of cellulase under SSF were optimized. Out of 60 fungal strains 6 fungal strains were positive for cellulolytic activity, only one positive fungal strain which showed clearer zone was selected for further work. The selected strain was named as *Penicillium brocae* PSKV2 after confirmation through automated genome sequencing. High enzyme activity was observed in jackfruit waste (23.96 U/mL) compared to corn cob (17.79 U/mL) and rice straw (11.40 U/mL). The optimum inoculum size for the production of cellulase enzyme was found to be 1mL showing enzyme activity of 27.38 U/mL at 5 days incubation period (15.06 U/mL), 65% moisture level (27.38 (U/mL), pH 6 (23.96 U/mL), and at 30°C temperature (27.38 U/mL). A novel fungal strain "*Penicillium brocae* PSKV2" was isolated from soil sample of decaying agrowaste dump in Bengaluru rural area. This pilot study finding could be explored in large scale industrial production of cellulase enzyme from cheaper agrowaste at the standardized optimum cultural conditions deduced in this study.

Introduction

Energy and environment are the essential aspects of human life almost all over the world. The conventional sources that meet the demand on energy needs will not last long and therefore non-conventional alternative and renewable sources are to be exploited for this purpose.[1,2] This planet is threatened due to environmental pollution in recent years as a result of disposal of solid and liquid wastes rich in organics. Solid and liquid waste rich in organics can be considered for generation of energy by biotechnological means.[3,4] Utilization of solid and liquid wastes will provide twin benefits saving the environment from polluted menace and generating energy.[5,6]

Lignocellulose is the most abundant organic compound in the biosphere; however, only a small amount produced in agriculture or forestry is used, the rest being considered waste, causing consequent deterioration of the environment.[7]

Much is being done to reduce losses of this resource and to diminish the resulting environmental degradation,[8] through the generation of a series of high-value products and byproducts such as cellulolytic enzymes and cellulosic ethanol.[9]

Cellulose constitutes bulk of the plant cell wall materials and is the most abundant and renewable non-fossil carbon source on earth.[10] Cellulose has enormous potential as a renewable source of energy,[11] and a number of microorganisms use it as a carbon source. Major constraints in enzymatic hydrolysis of cellulosic materials for the production of fermentation sugar are low productivity and the high cost of cellulases.[12]

Cellulase is a complex of three types of enzymatic complexes namely, cellobiohydrolases (EC 3.2.1.91), endoglucanases or CMCases (EC 3.2.1.4) and b glucosidases (EC 3.2.1.21), acting synergistically to convert complex carbohydrates present in lingocellulosic biomass into glucose.[13]

Recent interest by researchers to produce cellulase from cheaper substrate is justifiable. Choice of substrate is an important consideration in cellulase production. Different substrates have been employed in the production of cellulase, and they range from pure cellulose,[14] to diary manure.[15] Recently, agro residues have been used as carbon sources in cellulase fermentations. To make cellulase production economical, it has to be produced from readily available substrates and lignocellulolytic substrates offer this advantage. Many lignocellulosics have been reported for the production of cellulases. Amongst them include wheat bran, rice bran, corn cob,[16] corn straw, corn stalk and husks, sugarcane bagasse and cassava peels,[17-19] and saw dust.[20]

In addition to substrates, the fermentation technology applied can greatly determine the success of cellulase production. Different fermentation technologies exist for the production of cellulases; however, the popularly used technologies are submerged and solid-state fermentations. Solid state fermentation (SSF) by fungi is a preferable production route for cellulase as it offers lower cost and enables the production of cellulase with higher titre.[21]

Other advantages of SSF include maximum productivity, ease of technique, low capital investment, low energy requirement and less water output, better product recovery, and lack of foam build-up. Moreover, SSF has been reported to be most appropriate cellulase production process for developing countries.[22,23]

With this scenario, present study was conducted with the main purpose to investigate the production and optimization of cultural conditions for production of cellulase under SSF from *Penicillium brocae* PSKV2 using agrowastes such as surface-modified jack fruit waste and corncob as substrate.

Materials and Methods

Sample Collection and Isolation of Fungi for Cellulase Production

The soil samples that served as source of fungus used in this study were collected from the bottom of decaying agrowaste dumps from different locations of Bangalore rural area, Karnataka, India. One gram of soil was added in to100 mL of enrichment medium [g/L: Potato infusion – 20.0; Dextrose – 20.0, Yeast extract -10.0 and pH 5.0] in a 250 mL of Erlenmeyer flask and incubated at 28˚C for 5 days. A loopful of the enriched suspension was streaked on potato dextrose agar (PDA) plates and incubated at 28˚C for 5 - 7 days for the isolation of cellulolytic fungi. The fungal colonies were re-streaked on PDA for confirming the purity and transferred onto PDA slants and preserved for further use.

Screening of the Isolates for Cellulase Production

The fungal isolates were screened for cellulase production on screening medium, carboxyl methyl cellulose agar (CMC agar) [g/L: Cellulose – 10.0; $NaNO_3$ - 2.0; K_2HPO_4 - 1.0; $MgSO_4.7H_2O$ - 0.5; KCl - 0.5; $FeSO_4.7H_2O$ - 0.01; Agar - 20.0 and pH 6.0]. CMC agar plates were point inoculated with spore suspension of pure culture and incubated at 30°C for 5 days. After 5 days, the plates were checked for clear transparent zone by addition of 10 mL of 1% congo red solution for 10 mins after which excess congo red solution was decanted and later flooded with 1N sodium chloride for 15 minutes. The plates were observed for zone of decolorization around the colonies and diameter of the transparent zone around each colony was measured. Depending on the zone of decolorization, nine isolates PSKV2, PSKV5, PSKV9, PSKV12, PSKV14, PSKV15, PSKV18, PSKV21 and PSKV25 were selected for further experimental studies.

Evaluation of Agrowastes for Cellulase Production

Major regional agro wastes such as Jack fruit waste, corn cobs, paddy straw, sugarcane bagasse were collected from Bangalore rural district of Karnataka, India. All these agro-wastes were evaluated as substrate for the production of cellulase. The agro wastes were cleaned, dried in a hot air oven at 60°C for 10 mins and then blended in a blender at different particle sizes with a mean size of 1.0–2.0 mm and sterilized by autoclaving at 121°C for 15 min and then stored at 4°C until further use. Different type of agro wastes such as corn cobs (CC), jack fruit waste (JFW), paddy straw (PS), sugarcane bagasse (SCB) was supplemented individually into 250 mL of Erlenmeyer flask for cellulase production under SSF. The amount of cellulase produced was determined at every 24 h up to 120 h.

Corn cobs and jack fruit waste supported promising cellulase activity; hence these two substrates were selected for surface modification studies.

The alkali pre-treatment of substrates was conducted with 1: 10 ratio using 1 % (w/v) NaOH (1g of substrate in 10 mL of 1 % NaOH solution). Alkali pre-treated substrates were then washed with distilled water and neutralized to around pH 7.0 followed by drying.

Cellulase Assay

The culture was centrifuged at 10,000 rpm for 10 min at 4°C to obtain the cell free supernatant (CFS). The cellulase activity of the crude enzyme was determined by the modified method of Mandels and Weber (1971).[24] In brief, 0.5 mL of CFS was added to 0.5 mL of 1% carboxy methyl cellulose (CMC) as a substrate in 0.05 M citrate buffer (pH 4.8). The reaction mixture was incubated at room temperature for 30 min. The amount of reducing sugars released during hydrolysis was measured by DNS method. The reaction was stopped by the addition of 3 mL DNS reagent. The treated samples were boiled for 10 min and cooled in water to stabilize the colour. The optical density was measured using a UV-VIS spectrophotometer (ELICO SL-159) at 550 nm against the enzyme blank. The cellulase activity was measured using a calibration curve for glucose. In this study, one unit of cellulase was defined as the amount of enzyme required to release one µmol of glucose per mL per min under the above assay conditions.

Cellulase Production Under Solid State Bioprocess

The solid state bioprocess was evaluated using agro-wastes as follows: Five grams of JFW and CC was taken in 250 mL Erlenmeyer flasks and moistened (60 - 70 %) with sterile double distilled water. The contents were vigorously mixed, and the flasks were autoclaved at 121°C for 15 min. After sterilization, the flasks were cooled to 50°C and inoculated with 3 mL of *Penicillium brocae* PSKV2 isolate carrying 10^8 spores/mL. The flasks were incubated for 5 days at 30°C.

Further citrate buffer (0.1 M, pH 4.8) was added to the fermented substrate to a total volume of 100 mL and mixed for 1 h on rotary shaker. Samples were withdrawn at every 24 h of incubation and filtered through Whatman No. 1 filter paper to separate mycelial mat and culture filtrate. The supernatant was used as the crude enzyme for assay of enzyme activity.

Phenotypic and Genotypic Characterization of Potent Fungal Isolate

The phenotypic characterization of fungal isolate PSKV2 was carried out by mounting of viable culture and observed under microscope to determine its morphological characteristics. Genotypic

characterization of the fungal strain was carried out by sequencing of 18S rDNA at Europhins Lab Pvt. Ltd., Bangalore by automated genome sequencing. Further the obtained sequence was subjected to BLAST tool at NCBI to assign putative identity, designation of operational taxonomic units based on sequence similarity measures and phylogenetic inference. Duly annotated partial nucleotide sequence of the fungal strain was deposited with NCBI GenBank to procure accession number.

Neighbor joining analysis of fungal isolate PSKV2 mediating cellulase production and close relatives retrieved from GenBank using Clustal W. Phylogenetic tree was constructed with Molecular Evolutionary Genetics Analysis (MEGA) 6.0 software.

Results and Discussion

Isolation and Characterization of *Penicillium brocae* PSKV2

Out of 35 soil samples 60 fungal strains were obtained each fungal strain was cultured on freshly prepared potato dextrose agar medium.

Concurrently, Khatiwada *et al.,* also reported that cellulolytic fungi were isolated from soil which was later used for the production of cellulase enzyme.[25]

Out of 60 fungal strains 6 fungal strains were positive for cellulolytic activity, only one positive fungal strain which showed clearer zone was selected for further work (Figure 1).

The selected fungal strain was designated as PSKV2 and was stained using lactophenol cotton blue to study the morphology of fungi, by staining the positive fungal strain was found to be a species of *Penicillium*. In consistent with our study results, Khan *et al.,* also carried out in the similar protocol for the screening of cellulolytic enzyme.[26] Further confirmation of the PSKV2 strain was carried out by sequencing at Europhins Private Limited, Bangalore by automated genome sequencing, the PSKV2 strain was found to be *Penicillium brocae* (Figure 2). The strain was named as *Penicillium brocae* PSKV2. Similarly, the work, 16*S*rRNA gene sequencing and phylogenetic analysis of the CMC-degrading isolates was carried out by Liang *et al.,*[27]

Optimization of Culture Conditions for Cellulase Production Under SSF

High enzyme activity was observed in jackfruit waste (23.96 U/mL) compared to corncob (17.79 U/mL) and rice straw (11.40 U/mL). Hence jack fruit waste was used as the substrate for the production of enzyme (Figure 3).

Similar to this, in the work of Khan *et al.,* for the production of cellulase by SSF using substrates corn cob, newspaper waste, saw dust and wheat straw reported that corncob was suitable for the production of cellulase compared to other substrates.[26]

Optimization of inoculum size

The optimum inoculum size for the production of cellulase enzyme was found to be 1mL showing enzyme activity of 27.38 U/mL (Table 1), which supports the active growth of the fungi to produce the enzyme. In accordance with our study findings according to Chahal *et al.,* also the optimum inoculum size for the production of cellulase by SSF was 1mL.[28]

Optimization of incubation period

The optimum incubation period for the production of cellulase by *Penicillium brocae* PSVK2 was increased from 1st day to 5th day and the enzyme activity was decreased after 6thday. Hence the optimum incubation period for the production of enzyme was 5 days (Table 2).

In consistent with our study findings, according to the work of Amir *et al.,* incubation period for cellulase production by *Alternaria alternate* under SSF, corn cobs and sugarcane bagasse showed optimum incubation period on 5th day with maximum cellulase activity 15.06 ± 0.17 µg/mL.[29]

Optimization of moisture content

The optimum yield of cellulase using *Penicillium brocae* PSVK2 under SSF was obtained at 65% of moisture with the maximum enzyme yield of 27.38 U/mL (Table 3). In accordance with our study findings Kumar *et al.,* also reported optimum moisture level of 65% for the production of cellulase by SSF using *Trichoderma reesei*.[30]

Table.1 Effect of inoculum size on cellulase enzyme production

Inoculum Size (mL)	Activity (U/mL)
0.5	18.62
1.0	**27.38**
1.5	24.91
2.0	21.45
2.5	19.08
3.0	16.74

Table.2 Effect of inoculum period on cellulase enzyme production

Incubation Period (Days)	Enzyme Activity (U/mL)
1	12.48
2	16.92
3	21.67
4	25.84
5	**29.76**
6	26.10
7	22.34
8	18.59

Table.3 Effect of moisture level on cellulase enzyme production

Moisture Level (%)	Enzyme Activity (U/mL)
10	9.84
25	14.67
40	19.92
55	24.38
65	**27.38**
75	23.71
85	19.56
100	15.02

Table.4 Effect of pH on cellulase enzyme production

pH	Activity (U/mL)
1	5.42
2	8.76
3	12.94
4	17.38
5	21.65
6	**23.96**
7	21.08
8	18.42
9	15.16
10	12.03
11	9.47
12	7.12
13	5.06
14	3.24

Table.5 Effect of temperature on cellulase enzyme production

Temperature (°C)	Enzyme Activity (U/mL)
10	8.96
20	17.84
30	**27.38**
40	23.92
50	18.67
60	13.41
70	8.75
80	4.62

Figure.1 Selected positive strain of *Penicillium brocae* PSKV2 showing zone of clearance

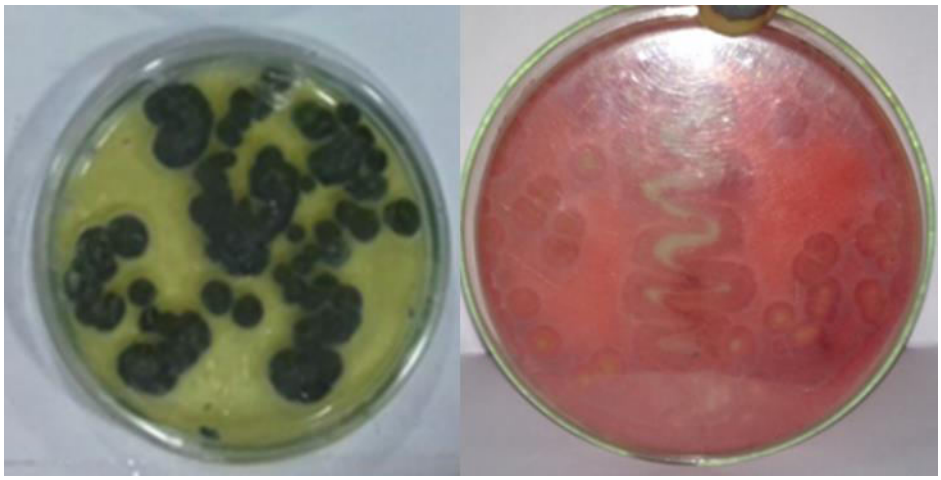
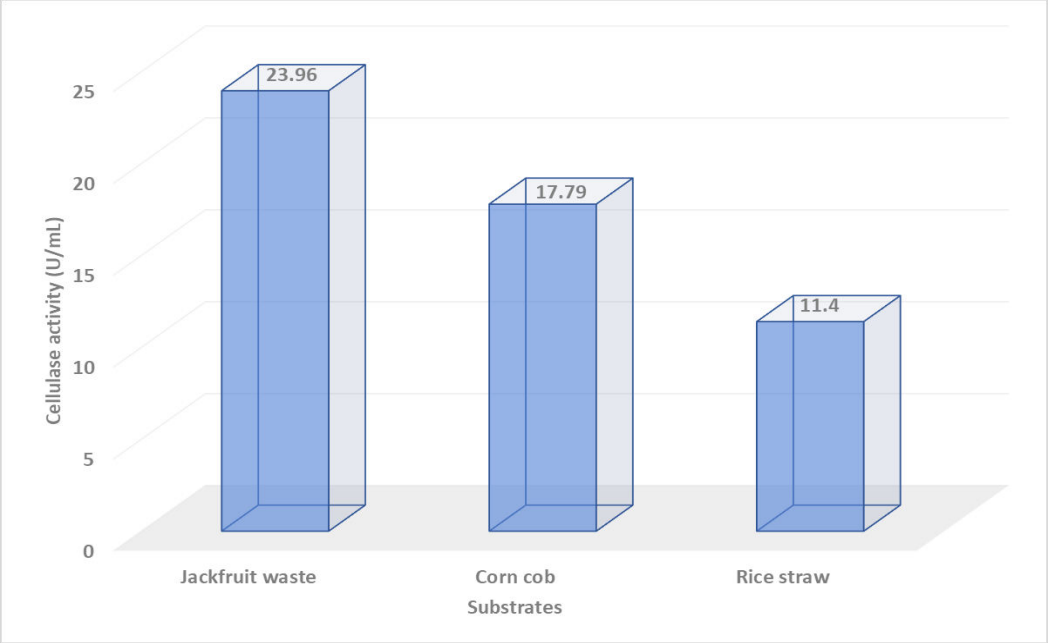


Figure.2 Genome sequence of *Penicillium brocae* PSKV2

>0518_058___PSKV_2_Consensus
ATTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGA
TCATTACTGAGTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTTTACCGTACC
CTGTTGCTTCGGCGCGCCCGCCCTCGCGGCCGCCGGGGGGCCTCCGCCCCCGGGC
CCGTGCCCGCCGAAGACACCCCTGAACGCTGTCTGAAGATTGTCGTCTGAGCGA
ATAGCGAAAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGA
TGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATC
ATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTC
CGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCGCCGTCCCCCCC
TTCCCGGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAG
CGTATGGGGCTCTGTCACCCGCTCTGCAGGCCCGGCCGGCGCCAGCCGACCCCCA
TCATCCTTTTTTTCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAA
GCATATCA

Figure.3 Screening of agrowastes for optimum cellulase production under SSF



Optimization of pH

The optimum pH for the effective production of enzyme was found to be at pH 6 with enzyme yield of 23.96 U/mL (Table 4). In concurrence with our study findings Khan *et al.,* also reported the optimum pH for the production cellulase by SSF using *Aspergillus niger* at pH 6.[26]

Optimization of temperature

The optimum temperature suitable for the growth of microorganism and production of cellulase enzyme at high rate using *Penicillium brocae* PSVK2 was found to be 30°C with cellulase enzyme yield of 27.38 U/mL (Table 5). Similar to this, Kumar *et al.,* reported the optimum temperature for the production of cellulase by

Trichoderma reesei under SSF was at 30°C temperature.[30]

In conclusion, a novel fungal strain "*Penicillium brocae* PSKV2" was isolated from soil sample of decaying agrowaste dump in Bengaluru rural area. The optimized culture conditions for the production of cellulase enzyme under SSF from *Penicillium brocae* PSKV2 using cheaper agrowaste as substrate were found to be with 1mL of inoculum size for 5 days of incubation period at 65% moisture, pH 6.0, and at 30°C temperature. This pilot study finding could be explored in large scale industrial production of cellulase enzyme.

Author Contributions

T. Vishwanatha: Investigation, formal analysis, writing—original draft. M. Keshavamurthy: Validation, methodology, writing—reviewing. A. C. Manjula:—Formal analysis, writing—review and editing. M. T. Ahalya: Investigation, writing—reviewing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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