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Biodegradation of lignocellulose from sugarcane bagasse by *Pseudomonas* isolates

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Abstract
In the present study investigated the biodegradation of sugarcane bagasse by Pseudomonas species isolated from sugarcane collected from land Pudukkottai District, Tamil Nadu. Pseudomonas was isolated and identified identified based on their morphological and biochemical charecteristics. The enzyme production by Pseudomonas sp was evaluated in different carbon sources: glucose; Lignocellulose degradation in sugarcane bagasse fortified with glucose was studied with and without ammonium sulphate. In bagasse fermentation, when the substrate initially containing 12.2% lignin was fortified with 5% glucose in the absence of ammonium sulphate, a maximum lignin loss of 5.4%, was recorded. This maximum lignin loss was accompanied by a minimum cellulose loss Efficiency of solid state fermentation using the sugarcane bagasse substrates indicates that lignin loss is high in comparison to cellulose loss.

Keywords: biodegradation, *Pseudomonas* sp and HPLC analysis

1. Introduction
In general, Lignin contains three aromatic alcohols (coniferyl alcohol, sinapyl and coumaryl). In addition, grass and dicot lignin also contain large amounts of phenolic acids such as P-coumaric and ferulic acid, which are esterified to alcohol groups of each other and to other alcohols such as sinapyl and P-coumaryl alcohols. Lignin is further linked to hemicelluloses and cellulose forming a physical seal around the later two components that is an impenetrable barrier preventing penetration of solution and enzymes.
There are many genera of actinomycetes and eubacteria which can degrade extracted lignin (Buswell and Odier, 1987). Many bacterial strains, especially actinomycetes, can solubilize and modify the lignin structure extensively, but their ability to mineralize lignin is limited (Buswell and Odier, 1987; Ball *et al.,* 1989; Eriksson *et al.,* 1990; Godden *et al.,* 1992) [14]. Actinomycetes degrade lignin as their primary metabolic activity at high nitrogen levels compared to white rot fungi, most of which degrade lignin via their secondary metabolism. Some species of *Streptomyces* and *Nocardia* have been shown to degrade some lignin in soil (Haider and Trojanowski, 1980).
Streptomyces badius mineralize 3.2^{14} C-MWL in 30 days, and mineralization was further enhanced to 11% when cellulose and yeast extract were added to the medium (Eriksson *et al.,* 1990) [14]. There are evidences that the grass lignins are attacked by actinomycetes more efficiently than the wood lignins (Buswell and Odier, 1987).
The carbon-carbon and ether bonds joining subunits together must be cleaved via an oxidative mechanism. Complex but non-specific enzyme systems are needed for lignin to be degraded. White-rot basidiomycetes tend to harbour gene families encoding the key enzymes responsible for deploymerisation of lignin manganese peroxidase (LiP), lignin peroxidase (MnP) and laccase. The existence of gene families for these extracellular enzymes may be a consequence of their diverse roles in fungal physiology. Lacccases. Have functions in plant pathogenesis, result of the non-uniformity of the substrate they oxidize.
"Hemicellulose", is the second component of lignocellulose, and they are mixtures of polymers made up of sugars (mostly not glucose) and sugar derivatives; the polymers may be branched and comprise different types of unit. A major component of lignocellulose in many types of plant (including trees, cereals and other grasses) in xylan. Xylan has a backbone of five-carbon sugar units (many of them acetylated), with side chains of sugar derivatives and is highly charged by virtue of acidic groups (sugar acids). Other hemicelluloses contain other sugars but, like the xylans, are all unordered, branched and charged. Hemicelluloses provide a matrix in which cellulose fibers are embedded to form the layered structure of plant cell walls.
Lignin, the third component present in lignocellulose, in the earth's most abundant aromatic plolymer and the most unusual of the lignocellulose polymers which is more recalcitrant to biodegradation. Delignification of lignocellulose by suitable chemicals treatments is an essential step before, it can be considered suitable for bioconversion to fermentable sugars or liquid fuels. Lignin is made up of units called lignols, each of which is aryl propanol composed of an aromatic ring and three carbon chain. The lignols are structurally very closely related to the amino acids phenylalanine and tyrosine from which they are ultimately derived.

2. Objectives
1. To collect the soil sample from garden soil
2. To isolate and identify the bacteria using standard methods
3. To screen the organisms for Lignocelluloses degradation

4. To optimize the Lignocelluloses degradation by physicochemical parameters
5. To analyze the enzyme production

3. Materials and Methods

Collection of Sample

Soil and sugarcane bagsse were collected from Agricultural land, Mangadu, Pudukkottai district, Tamil Nadu.

Isolation and Identification of *Psendomonas species*

Psendomonas was isolated and were identified on the basis of their morophological and biochemical characteristics according to Bergey's manual of Determinative bacteriology (Holt *et al.,* 1994).

Enumeration of the microbial populations

Samples of sugarcane bagasse were separately collected from several points,mixed and transported to the laboratory and immediately used for the analysis of total Colony Forming Units (CFU) of bacteria, fungi and actinomycetes and were enumerated using standard plate count method, (Daniel and Karmegam 2000; Parthasarathi and Ranganathan, 1998 and Subbarao, 1995). Several microbial colonies of bacteria, fungi and actinomycetes were growth in the respective medium, several bacterial isolates which showed predominant growth were selected for further studies

Preparation of medium

The medium was peptone cellulose solution composed of 5g peptone, 10g cellulose (rice stalk), 1g yeast extract, 3g $CaCO_3$, and 5g NaCI in 1 L of H_2O (pH 8.0). For sterilization, the medium was autoclaved at 121^0c for 20 min.

Analytical methods

Cellulase was produced in modified Mandels Weber medium (1969), adapted to receive different carbon sources, glucose (Mandels-Weber basic medium; G), non-treated bagasse (BNT), bagasse treated with 4% solution of sodium hydroxide (BSN), bagasse treated with 4% solution of sodium hydroxide phosphoric acid-steam (BSPH), and filter paper (FP). Bacterial suspension was obtained by addition of distillated sterilized water (10 mL). Five milliliters of this suspension was inoculated with 200-mL liquid medium that was incubated at 30ºC and 100 rpm, in circular agitator. Samples were analyzed daily and stirring at 6000 x g during 15 minutes to evaluate the enzymatic activity, according to Mandels (1974).

The pretreatments were: BSN: 100 g of the washed and ground sugarcane bagasse were treated with 2000 mL of 4% solution of sodium hydroxide, autoclaved at 121ºC for 30 minutes. The material recovered by filtration was washed with distilled water until neutrality and dried at 65ºC to constant weight; BSPH: one portion of the bagasse obtained in BSN, before the successive washes with water, was neutralized with phosphoric acid and filtered. The material recovered was then dried at 65ºC to constant weight. To the bagasse obtained, the same volume of distilled water was added and heated at 121ºC for 30 minutes. The suspension was filtered and the solid material dried at 65ºC to constant weight.

The cellulose percentage in the bagasse used was evaluated according to Updegraff (1969). Ground sugarcane bagasse was incubated to 100ºC with nutricacetic solution (80% acetic acid and 100% acid nitric) for 30 minutes. After centrifugation to 3000 x g for 60 minutes at room temperature, a solution of 72% sulfuric acid was added to the precipitate. To evaluate the cellulosic percentage was mixed 1 mL of sample, 8 mL of 2% anthrone solution and 4 mL of distilled water, being this solution incubated in boiling water for 15 minutes. The spectrophotometric measuring was made against curve cellulose (g/mL, w/v) to 620 nm. The lignin was certain in agreement with Milagres et al. (1994), being considered as lignin the remaining solid residue after the hydrolysis with sulfuric acid at 72% and powder-hydrolysis with sulfuric acid at 4%. The residue was filtered and washed with water distilled to remove the acid sulfuric, being maintained even in stove for 105ºC constant mass. The lignin was expressed by the following equation:

$$\% \text{ of lignin} = \frac{\text{Lignin weight (g)}}{\text{Bagasse weight(g)}}$$

Optimization of physicochemical parameters in Sugarcane bagasse biodegradation

Effect of temperature on cellulase activity

The effect of the temperature on the cellulase activity was determined by incubating 1 mL of crude extract of cellulase with 50 mg of filter paper Whatman No. 1 during 1 hour at 30, 40, 50, 60 or 70ºC. The enzymatic activity was determined for all temperatures. The thermostability of cellulase was determined incubating the cellulolytic extract in the temperatures of 30 to 70ºC. The residual enzymatic activity was evaluated after 30, 60, 120, 180 and 240 minutes, according to Mandels method (1974). The results were obtained by triplicate.

The cellulase activity was obtained according to Mandels (1974). The concentration of reducing sugars produced (glucose) was determined by Miller method (1959). One unit of cellulase corresponds to the amount necessary to form 1 mmol of glucose per minute at 50ºC.

The influences of three factors in the hydrolysis of the cellulose from sugarcane bagasse were studied using a factorial design constituted of 23 experiments with three repetitions. To this experiment the influences of three variables were considered: the bagasse type (A), enzyme (B) and temperature (C).

Effect of substrates on fermentation

Sugarcane bagasse and rice straw were chopped into 30-50 mm pieces and were separately soaked in boiling water for 10 min and the excess water was drained. About 25 g of moistened bagasse and rice straw were separately transferred into 1000 ml Erlenmeyer flasks and used as the biodegradation substrate. To each flask was added, 10 ml of nutrient medium containing 5% glucose or glucose and 0.21% ammonium sulphate. Culture medium not fortified with glucose and without ammonium sulphate served as the control substrate. Flasks contain the substrate were autoclaved at 120ºC. C and 1.06 kg/ cm 2 for 15 min and the fermentation was carried out at 25 C for 21 days. The fermented and untreated substrates were oven dried at 100-105 C, and ground

to less than 1 mm pieces for determination of cellulose and lignin.

Determination of cellulose to 1 g of dried sample, 15 ml of 80% acetic acid and 1.5 ml of conc. nitric acid were added and refluxed for 20 min. It was filtered and the residue was washed with ethanol, dried in an oven at 100-105 C and weighed (Material A). It was then incinerated at 540 C (Material B). Cellulose content was determined according to the method of Van Soest and Wine (1967) [45]. All experiments were carried out in triplicates.

Percentage of Cellulose (Material A) (Material B) / Weight of the sample x 100

Determination of lignin

To 1 g of dried sample, 70 ml of 1.25 sulphuric acid was added. The mixture was refluxed for 120 min, filtered and washed in water. To this was added 30 ml of 72 sulphuric acid and the material was allowed to stand for 4 h with occasional stirring. It was then filtered, washed and dried at 100-105 C (Material A) and incinerated at 540 C (Material B). Lignin content was determined according to the procedure of Van Soest and Wine (1967) [45]. All experiments were carried out in triplicates. Percentage of Lignin (Material A) (Material B)/Weight of the sample x 100

Statistical analysis

All analysis was performed in triplicate and results were presented here by the mean of triplicate ± standard deviation (SD).

HPLC Analysis

Column Specification

Reverse phase HPLC (Cyberlab,USA) analysis was carried out in a C 18 column (250mm ×4.6mm) version (lake forest,CA USA)equipped with a c 18 curved column. The components were eluted with an isocractic elution of acetonitrile vs water at the flow rate of1 ml /min and absorption recorded at 680nm.

Sample preparation

One ml of the samples was centrifuged (at3000rpm for 15 minutes) and dissolved in specific solvent of HPLCgrade and filtered through 0.22 micro filter. The filtrate was collected and degassed using sonicator for 50 times at 4°c.

Solvent preparation

Solvent was prepared using aceto nitrile and water in the radio 65:35 and degassed using sonicator for 15 times at 4°c.

Column equilibration

Column equilibration was done using 65% aceto nitrile in water until zero base line.

Sample injection

Twenty micro litre of the sample was injected in to the injection head using injection needle. Required time and wavelength were set and the purification profiles were seen on the screen that shows the degraded components with its retention time

4. Results and Discussion

In the present study, *Pseudomonas sp* isolated from soil by dilution plate method and it was identified based on their morphological and biochemical characteristics according to the Bergey's Manual of Systematic bacteriology.

Isolation of bacteria from soil

This organism was isolated by repeated subculture in liquid medium and selection of single colonies from agar plates containing.

Description

The organism is a Gram-negative rod measuring 0-8, u x 2-0 u and is motile with a single polar flagellum (Fig. 1). Colonies on agar are pink, raised, moist, circular and have smooth edges. They are pin-point within 2 days and reach a maximum diameter of 1 mm. after 1 week. The organism is strictly aerobic. Unshaken cultures produce a thin pellicle and shaken cultures have a tendency to clump which is particularly marked in growth on methylamine hydrochloride.

Composition of Untreated Sugarcane Bagasse

Untreated Sugarcane Bagasse mainly consisted of carbohydrates (cellulose and hemicelluloses),lignin and extractives in respective degrees Lignin removal of pretreated Sugarcane Bagasse was computed with respect to total lignin referred to summation of Klason lignin (acid insoluble lignin) and soluble lignin of untreated SCB

Production of Cellulase

Figure 1 showed the effect of the cellulose percentage in the enzymatic production from *Pseudomonas sp*. Cellulolytic activities about of (0.8 UI mL-1), with filter paper and (0.7 UI mL-1), with BSN were obtained after 192 hours. The lignin content was important in the cellulase production. The cellulose percentage was greater in the BSPH-treatment than in the BSN-treatment, although had sensible reduction in the enzymatic activity, suggesting that the greater lignin percentage into BSPH will could be associated with this reduction.

Optiomization of physicochemical parameter Effect of the temperature on enzymatic activity of *Pseudomonas sp*.

The effect of the temperature on the activity and the stability of an enzymatic extract is important in biological processes. With this purpose, the effect of the temperature on crude extract of cellulase from *Pseudomonas sp* was evaluated. The cultivation was started in Mandels-Weber medium using filter paper as carbon source. The enzymatic activity increased with the increase of the temperature until approximately 60ºC (0.8 IU mL-1) and decreasing at 70ºC

The values in the literature such as cellulase from *Trichoderma reesei* ITCC-1433 (Herr, 1980), cellulase from *Trichoderma viride* QM-9414 (Iaderoza and Draetta, 1984), cellulase from *Bacillus circulans* (Trevino *et al.*, 1989; Jana *et al*. 1994; Kaur *et al.* 1998 were evaluated) [44, 23, 27]. Vitti (1988) verified that at 45ºC obtained greater cellulolytic activity from *Aspergillus sp*., which was also was observed.

When used cellulases from *Neocallimastix frontalis*. However, values observed for this enzyme when immobilized were higher, suggesting that will be more stable and recyclable

Effect of carbon and nitrogen sources on degradation and fermentation of sugarcane bagasse by *Pseudomonas sp*

Cellulose and lignin content in untreated sugarcane bagasse was 54.2% and 17.3% respectively Fermentation of unfortified baggase by *Pseudomonas sp* for 21 days resulted in 11.5% of cellulose and 12.2% of lignin. Supplementation of sugarcane bagasse with 5% glucose resulted in a decrease of 2.0% in cellulose and 23.7% of lignin. Addition of sugar helped in the hydrolysis of lignin. A reduction in the loss of cellulose when 5% glucose was added to the fermentation medium indicated that *Pseudomonas sp* utilized glucose instead of cellulose as a source of energy for fungal metabolism including the production of hydrolytic enzymes.

Addition of 0.21% ammonium sulphate to the medium containing 5% glucose and sugarcane bagasse showed a reduction of 7.8 %cellulose and 5.4% of lignin These results agree with Odier and Roch (1983) [38], who reported that degradation of poplar wood, by white rot fungi, was stimulated by reduced glucose concentration. Addition of 0.21% ammonium sulphate to the medium containing 5 glucose and sugarcane bagasse showed a reduction of 8.89% cellulose and 6.9% of lignin. Fermentation of rice straw under similar conditions resulted in a reduction of 3.4% cellulose and 11.4% lignin. Lignocelluloses biodegradation by Ander and Eriksson (1997) also reported that urea, casemic acid and ammonium phosphate gave the lowest lignin degradation. Sources of nitrogen have been reported to repress the degradation by *P. chrysosporium* (Fenn and Kirk, 1981) [15]. The ability of basidiomycetes to recycle their own nitrogen has been reported by (McDonald *et al.,* 1977) [33]. The most extensive lignin degradation was observed when glucose was supplied to nitrogen starved condition where Termitomyces sp. preferentially degraded lignin in the presence of 5 glucose. Similar results have been reported previously (Jalk *et al.,* 1998) [22]. Results reported here were in agreement with those of Odier and Roch (1983) [38] who reported that degradation of C14 labeled poplar wood, by several white rot fungi, was stimulated at limiting glucose concentration. Abdullah *et al.* (2004) [13] indicated that lignin hydrolysis was best at a marginal carbon/energy source for maintenance of metabolism. Decomposition of lignin was reported to take place after profuse microbial growth. It was further reported that no microorganism could grow on carbohydrate-free medium and decomposition of lignin was difficult in the absence of readily degradable, high-energy source (Costa *et al.,* 2002) [12]. In conclusion, rice straw, the major agricultural by-product of South Asia, is high in lignin and cellulose than sugarcane bagasse following degradation through solid state fermentation.

HPLC analysis

The absorption spectra of the samples obtained at 300nm are presented in figures 3 and 4. The sugarcane bagasse control showed 2 peaks with retention time (RT) of 4.616 and 9.858 for 30 minutes. The elution profile obtained for the bacteria treated samples significantly different from the control in terms of number, height of peaks obtained and RT.The HPLC profile of sugarcane baggers treated with bacterial isolate pseudomonas showed 3 peaks with RT 4.281, 8.008 and 10.208.

Table 1: Effect of the cellulose and lignin on the cellulolytic activity of *Pseudomonas sp*

S. No	Bagasse type	% of cellulase	% of lignin	cellulase
1	G			0.08
2	BNT	60	5	0.2
3	BSN	50	25	0.28
4	BSPH	65	10	0.7
5	FP	100	3	0.8

(G) glucose; (BNT) non-treated bagasse; (BSN) bagasse treated with 4% NaOH; (BSPH) bagasse treated with 4% NaOH-H3PO4-steam; (FP) filter paper.

Table 2: Lignocellulose degradation of sugarcane bagasse with nutrient medium fortified with nitrogen and carbon sources during solid-state fermentation by *Pseudomonas sp* for 21 days at 25° C.

S. No	Substrate fortification	Bagasse analysis g/100g		Loss in constituents (%)		Digestability (%)
		Cellulose	Lignin	Cellulose	Lignin	
1.	Fresh bagasse not fermented	54.2±0.3	16.2 ± 1.3			10.5±0.3
2.	5 % Glucose	52.2±1.0	12.1 ± 0.2	2.0	23.7	23.3±2.1
3.	5%Glucose +0.21%$(NH_4)_2$ SO_4	49.2 ± 0.2	15.2 ±1.0	7.8	5.4	11.8±1.0
4.	Not fortified	43 ± 0.3	14.1 ±1.0	11.5	12.2	17.3±0.5

Culture medium consist of 0.3g MgSO4.7H2O, 2gKH2PO4, 0.4g CaCl2 2H2O,0.1g yeast extract made to litre.

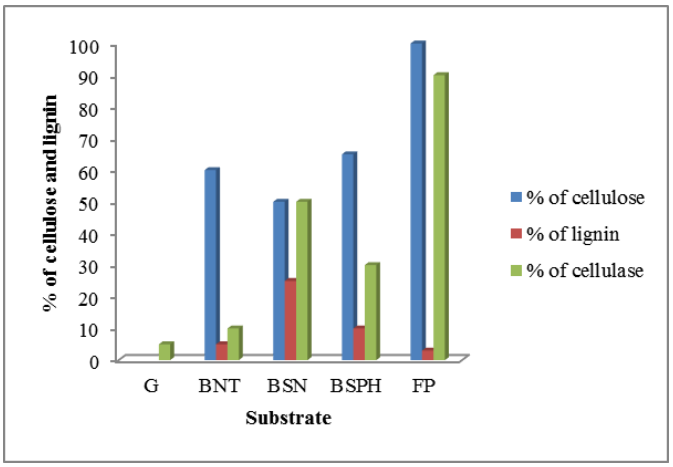

Fig 1

5. Conclusion

The current study emphasizes the possible benefits from the application of cellulase from *Pseudomonas* in the conversion of cellulosic materials when compared with commercial enzyme, with same activity. Sugarcane bagasse showed to be a possible inductor for enzyme synthesis, due its cellulose content. Maximum enzymatic activity was obtained with filter paper (0.8 IU mL-1), which possesses minimum amount of lignin, that usually forms a barrier for cellulases.

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