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ISOLATION AND CHARACTERIZATION OF CELLULOLYTIC BACTERIA FROM DECOMPOSING RICE STRAW

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Abstract: Three cellulolytic bacterial strains were isolated from decomposing rice straw. They were able to utilize cellulose, rice straw powder and carboxymethylcellulose as substrates. Two of these strains, *Listeria* sp. and *Enterobacter* sp., were abundant during initial stages of decomposition whereas the other strain, *Pseudomonas* sp., became dominant towards the late stages of the process. Comparison of their endo-1, 4- β -glucanase (carboxymethylcellulase) activity by indirect methods indicated a higher level of enzymatic activity in the *Pseudomonas* sp. than in the other two strains. Release of glucose by saccharification of cellulose and carboxymethylcellulose was also higher with *Pseudomonas* sp. compared with *Listeria* sp.

Key words: Cellulase, cellulolytic bacteria, lignocellulose, rice straw.

INTRODUCTION

Most agricultural residues of crop plants, particularly cereals, are rich in lignocellulosic materials.^{1,2} Cellulose, a long-chain polysaccharide made of $\beta(1,4)$ -linked glucose units, is the principal constituent of lignocelluloses. Association of cellulose with lignin, another complex polymeric molecule composed of phenylpropanoid units, forms the lignocelluloses. Hemicellulose is the other major component of lignocelluloses. It is a heterogeneous group of long-chain polysaccharides of which basic units are arabinose, xylose, mannose or galactose. Degradation of lignocellulosic material is a slow process and only a relatively narrow taxonomic range of bacteria and fungi are able to degrade such material. The ability of microorganisms to degrade cellulosic material is of considerable interest both in terms of microbial ecology and biotechnology.

Degradation of cellulosic material requires the cooperative action of a family of cellulolytic enzymes that have been classified into three major groups: endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91) and β -glucosidases (EC 3.2.1.21).³ Cellulolytic properties of these enzymes have been studied mostly in fungi.⁴ The bacterial cellulase system has only recently become a focus of investigation. Due to high cellulolytic activity of some bacteria and their short generation time they are a more promising group of organisms in degrading lignocellulosic wastes.⁵ In this paper we report isolation and characterization of three cellulolytic bacterial strains involved in the degradation of rice straw, a widely available agricultural residue in Sri Lanka.

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METHODS AND MATERIALS

Isolation of bacteria from rice straw

Rice straw samples representing different stages of the process of decomposition, starting from one week after harvesting, were used for this study. Each sample (5g) was washed with 15 ml of sterile 0.07 M potassium phosphate buffer, pH 7.2, with vigorous shaking and the washing was filtered through two Whatman 3 MM filter papers. From each filtrate a dilution series (10^{-1} - 10^{-4}) was prepared using sterile potassium phosphate buffer, pH 7.2, and 0.1ml samples were spread on L- agar (10g tryptone, 5 g yeast extract, 10 g NaCl, 1g glucose and 15g agar per liter)⁶ plates and incubated at 37°C.

Identification of cellulolytic bacteria

Different types of bacteria from L-agar plates were inoculated onto the cellulose agar (2.5 g of precipitated cellulose, 0.5g peptone, 0.2 g dipotassium-hydrogen phosphate, 0.2g magnesium sulphate, 0.4 g potassium carbonate, 0.02 g calcium chloride, ferrous sulphate and sodium chloride, and 15g Oxoid ion agar per liter) and incubated at 37°C. Only the bacterial strains capable of degrading cellulose grow well on this medium. None of these strains grew on the agar medium without cellulose. Thus, several cellulolytic bacterial strains were isolated and on the basis of morphological and biochemical characteristics they were identified to the genus level.

Confirmation of cellulolytic activity

Bacterial strains identified as cellulase positive were further tested for their cellulolytic activity by growing in media containing rice straw powder or carboxymethylcellulose (CMC) (BDH, high viscosity), a semisynthetic cellulosic substrate.

Visualization of CMC (β -D-Glucan) hydrolysis

One of the enzymes required to convert cellulose to glucose is endo-1,4- β -glucanase,⁷ which can be detected by the hydrolysis of CMC. Enzymic activity of the isolated bacterial strains was visualized as described⁸ with a slight modification. Inoculum from each fresh bacterial culture was patched on CMC agar which contained CMC (1 mg/ml) instead of cellulose in the cellulose agar medium, and incubated at 37°C for 8h. Plates were then flooded with an aqueous solution of congo red for 15min. After pouring off the congo red solution, plates were flooded with 1M NaCl for 15 min. After that zones of hydrolysis could be seen as clear areas.

Release of glucose by saccharification of cellulose and CMC

Cellulose broth containing 0.5% cellulose (w/v) and all the other ingredients except agar found in the cellulose agar medium was used to determine the

saccharification of cellulose. CMC broth was prepared using 0.5% CMC (w/v) instead of cellulose. Samples of bacterial cultures (0.5 ml) grown in these broths to a same cell density were used to inoculate 50ml of fresh broth in 250 ml flasks. These cultures were incubated at 37°C with continuous shaking, for 24 h, and then centrifuged at 5000 g for 10 min. Glucose concentration of the supernatants were determined as described.⁹ A 0.1 ml sample of the supernatant was boiled with 5 ml *o*-toluidine for 10 min, and OD₆₃₅ was determined. Corresponding glucose concentration was read from a standard calibration curve of glucose concentration (0 - 15 mmol/l).

RESULTS

L-agar plates prepared with diluted straw-washings were used initially to determine different types of bacteria present at various stages of decomposition (sampling period was from one week after harvesting to three months). In all samples there were three prominent types of bacteria that could be distinguished by their colony characteristics. Only these strains grew on cellulose agar demonstrating their cellulolytic activity. Morphological and biochemical characteristics showed that these strains belong to the genera *Pseudomonas*, *Listeria* and *Enterobacter* (Table 1).

Table 1: Characteristics used to identify the bacterial strains

Characteristics	<i>Listeria</i> sp.	<i>Enterobacter</i> sp.	<i>Pseudomonas</i> sp.
Gram's reaction	G ⁺	G ⁻	G ⁻
Cell shape	Irregular rods	Rods	Rods
Motility	+ ^a	+	+
Oxidase	- ^b	-	+
Acid production from sucrose	+	+	+
lactose	+	+	+
galactose	+	+	NT ^c
arabinose	+	NT	NT
maltose	NT	+	NT
mannitol	NT	+	NT
Methyl Red test	+	+	+
Urease	-	-	-
Voges-Proskauer test	-	-	-
Indole test	+	+	+
Nitrate reduction	+	+	+
Starch hydrolysis	+	NT	-
Utilization of citrate	NT	+	NT
Growth in KCN	NT	+	NT
Cellulose hydrolysis	+	NT	NT

a - Positive result or growth, b - Negative result or no growth, c - Not tested.

These three strains were present in similar numbers in the straw samples which were less than one month old. However, in the samples taken from straw

older than one month, *Pseudomonas* sp. was abundant while the numbers of the other two strains gradually decreased (Table 2).

Table 2: Bacterial counts in straw-washings prepared from straw in different stages of decomposition.

Age of the straw sample	No. of cells/ml ^a		
	<i>Pseudomonas</i> sp.	<i>Listeria</i> sp.	<i>Enterobacter</i> sp.
1 week	4.5 x 10 ⁴	4.8 x 10 ⁴	5.0 x 10 ⁴
1 month	4.7 x 10 ⁴	4.5 x 10 ⁴	3.8 x 10 ⁴
2 months	2.5 x 10 ⁴	2.2 x 10 ⁴	2.0 x 10 ⁴
3 months	6.0 x 10 ⁵	1.8 x 10 ³	1.0 x 10 ³

a - Average of the number of colonies in plates inoculated with samples from a serial dilution (10⁻¹ - 10⁻⁴) of straw washings.

All three strains grew well in the media containing CMC, and rice straw powder, confirming their ability to utilize these cellulosic substrates. The Congo red method used to visualize the hydrolysis of CMC enabled comparison of the endo-β-1,4-glucanase activity of the three strains. *Pseudomonas* sp. produced the largest zone of hydrolysis (Fig. 1A) (clear zone) indicating a high level of enzymatic activity. Zones of hydrolysis produced by *Listeria* sp. (Fig. 1B.1) and the *Enterobacter* sp. (Fig. 1B.2) were similar in size, but they were smaller than the zones produced by the *Pseudomonas* sp. This suggests that the level of their enzymatic activity is similar to but less than that of *Pseudomonas*.

Figure 1: Endo-1,4-β-glucanase activity of the isolated bacterial strains visualized as clear zones of hydrolysis. Bacterial colonies were grown on CMC agar and then plates were stained with Congo red to visualize the clear zone. (A) *Pseudomonas* sp, (B.1) *Listeria* sp, (B.2) *Enterobacter* sp.

Release of glucose by the saccharification of cellulose and CMC was examined only with the *Pseudomonas* sp. and *Listeria* sp. *Pseudomonas* sp. released 2.0 and 1.8mmol/l glucose when cellulose and CMC were used respectively. Concentrations of glucose produced by *Listeria* sp. were 1.8mmol/l with cellulose and 1.5mmol/l with CMC.

DISCUSSION

Cellulolytic bacterial strains isolated from decomposing rice straw were able to utilize both natural and semi-synthetic cellulosic substrates. Variation of the frequency of occurrence of these three genera with time is evidence of existence of a cellulolytic bacterial succession on this substrate. The role of *Listeria* sp. and *Enterobacter* sp. seems to be more important during the early stages of decomposition. Increasing frequency of the *Pseudomonas* sp. after about one month suggests that the action of the enzymes produced by this strain is required during the later stages. As detected from the Congo red method, this *Pseudomonas* sp. has a high level of endo-1,4- β -glucanase activity. This is one of the enzymes required for the conversion of cellulose to glucose. Saccharification of wheat straw yields a number of products including reducing sugars xylose and glucose.¹⁰ Since glucose or its oligomers have not been found in cereal straw hemicellulose fractions,¹¹ the production of glucose confirms hydrolysis of the cellulose component of straw. Release of high amounts of glucose from both cellulose and CMC by *Pseudomonas* sp. compared to the amounts of released glucose with *Listeria* sp. confirms its high level of production of extracellular cellulases. *Pseudomonas* sp. with cellulase activity has been isolated previously.¹² *Pseudomonas* sp. isolated in the present study appears to have high cellulase activity in comparison with the bacteria isolated earlier¹² and may be suitable for genetic manipulation to achieve a higher efficiency of degradation of rice straw.

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