American-Eurasian J. Agric. & Environ. Sci., 12 (1): 128-132, 2012
ISSN 1818-6769

Effect of *Bacillus pumilus* EWBCM1 Whole Cell Immobilization in Various Matrices on Cellulase Enzyme Production and Saccharification of Sugarcane Bagasse

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Abstract: The purpose of this investigation was to study the effect of immobilized *Bacillus pumilus* EWBCM1 cells in various matrices such as calcium alginate, gelatin, agar-agar, polyacrylamide, scotch brite and polyurethane foam on the production of cellulase enzyme and saccharification of sugarcane bagasse. Scotch brite was found to be an effective and suitable matrix for maximum cellulase productivity (0.603 ± 0.03 IU/ml) compared to the other matrices studied ($p<0.05$). The maximum reducing sugar (1.63 ± 0.04 mg/ml) was released by *Bacillus pumilus* EWBCM1 immobilized cells on scotch brite. All the above mentioned these matrices were applied for repeated batch fermentation. The scotch brite immobilized cells of *Bacillus pumilus* EWBCM1 used for four batches, it had significant ability for cellulase production and for saccharification of sugar cane bagasse. It has been shown that the immobilized cells were consistently able to produce the enzyme and they might be suitable for continuous enzyme production.

Key words: Cellulase · Bacillus pumilus EWBCM1 · Immobilization · Repeated batch fermentation

INTRODUCTION

Enzymatic hydrolysis for cellulosic wastes utilization could be accomplished through a complex reaction of various enzymes. Cellulases are inducible enzymes, which are synthesized by microorganisms during their growth on cellulosic materials [1]. Therefore, very much researches aimed at obtaining new cellulase producing microorganisms with greater efficiency were carried out. Bacteria, which have high growth rate comparing to fungi has good potential for cellulase production. Bacterial cellulase can be applied to great extent in different scopes, e.g. economical production of ethanol and other chemical industries, food and pharmaceutical industries [2].

Cell immobilization compared to the free cell systems, has a wide research interest according to various advantages. Although the immobilized cells achieve high rates of operation, in general, the applied supports used for cell immobilization in food production must of food grade purity another cheap and abundant [3]. The efficiency of immobilized cells was stable from batch to batch and they were suitable for industrial fermentations [4]. Immobilization is very important for commercial uses, where the immobilized enzyme can be reused and easily removed from the hydrolyzed mixture. The immobilization process may or may not change the general properties of the immobilized enzyme [5].

Whole cell immobilization by entrapment is a widely used and simple technique. The success achieved with the entrapment technique prompted the study of the cellulase production with immobilized cells, using this technique [6]. The natural polymers such as agar, agarose, pectin and gelatin were also employed for cell immobilization [7].

Sugar cane bagasse is an industrial waste containing more than 30% of cellulose, is a more abundant source and available in local area and could be used as substrate for the production of glucose, microbial proteins and cellulase enzymes. Sugarcane bagasse was utilized as suitable substrate for the production of considerable amounts of cellulase enzymes by *Penicillium varabili, Cellulomonas, Trichoderma ressi* [8]. As India is one of the largest sugarcane producing country, molasses, a byproduct of sugar cane industry is mostly used as a raw material for fermentation [9]. The digestibility of lignocellulosic biomass during enzymatic hydrolysis is

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hindered by its structural features such as high cellulose crystallinity, lignin content, hemicellulose acetylation and inaccessible surface area [10].

Enzymatic hydrolysis of cellulose is carried out by celullase enzyme system and the products of hydrolysis are usually reducing sugars including glucose. Utility cost of enzymatic hydrolysis is low as compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45-50°C), with no corrosion problem. Both bacteria and fungi can produce cellulase for performing the lignocellulosic materials hydrolysis [11]. The present investigation studies the effect of immobilization of *Bacillus pumilus* EWBCM1 cells on various matrices effect on the production of cellulase enzyme system suitable for the sugar cane bagasse saccharification.

MATERIALS AND METHODS

Cellulose Hydrolyzing Bacteria: Cellulose hydrolyzing bacterium *Bacillus pumilus* EWBCM1 was obtained from Microbiology Laboratory, Ayya Nadar Janaki Ammal College, Sivakasi, Tamil Nadu, India. The culture was maintained on carboxy methyl cellulose- agar slants and stored in refrigerator at 4°C [12].

Lignocellulosic Waste: In the present study, sugar cane bagasse was used as a substrate for cellulose hydrolysis by the bacterial cellulose system, it was collected from the sugarcane plantation area of Sivakasi, Virdhunagar district, Tamil Nadu, India. For pretreatment the waste, it was milled into small particles (sieved to 40 mesh) and air dried at room temperature, also was pretreated by steam explosion, as recommended for lignocellulosic materials pretreatment [13].

Effect of Whole Cell Immobilization by Entrapment in Various Matrices on Saccharification Process

Preparation of Cell Suspension: *Bacillus pumilus* EWBCM1 was grown in the carboxy methyl cellulose-agar medium. Cells after incubation were harvested by centrifugation at 10,000 rpm for 15 min. Cells pellet were washed with 2% (w/v) KCl solution, followed by 0.9 % (w/v) NaCl solution and sterile distilled water subsequently. Finally cells were suspended in NaCl solution and stored at 4°C for further studies.

Entrapment in Different Supports

In Calcium Alginate: Sodium alginate solution (3% w/v) was prepared in boiling water and autoclaved at 121°C for 15 min. Both alginate slurry and cell suspension (2 ml/10ml of alginate) were mixed and stirred for 10 min to get a uniform mixture. The slurry was taken into a sterile syringe and added dropping into 0.2 M $CaCl_2$ solution from 5 cm height and kept for curing at 4°C for 1hr. The cured beads were washed 4 times with sterile distilled water [14].

In Polyacrylamide: Cell suspension (2 ml) was added in 10ml of chilled sterile distilled water. To another 10 ml of 0.2 M sterile citrate phosphate buffer (pH 7.0) contains the following Compounds (g): acrylamide-2.85 g; bisacrylamide-0.15 g; ammonium per sulphate-10 mg and TEMED-1ml. Both solutions were mixed well and poured into sterile flat bottom 10 cm diameter Petri-dish. After solidification the acrylamide gel was cut into equal size (8 mm), transferred to 0.2 M phosphate buffer (pH 7.0) and kept in the refrigerator (1hr) for curing. The polacrylamide gel was washed 4 times with sterile distilled water [15].

In Agar-Agar: Agar-agar solution (2% w/v) was prepared in (0.9% w/v) NaCl solution and autoclaved at 121°C for 15 min. Cell suspension (2 ml) was added to molten agar-agar maintained at 40°C, shaken well for few seconds (without forming foam) poured into sterile Petri plates. The solidified agar block was cut with well puncture (8 mm) added to sterile 0.1 M phosphate buffer (pH 7) and kept in the refrigerator (1hr) for curing. After curing, the agar blocks were washed 4 times with sterile distilled water [16].

In Gelatin: Cell suspension (2 ml) was added to 15 ml of (20% w/v) sterile gelatin, maintained at 45°C and poured into a sterile Petri plates. The gel was over layered with 10 ml of (5% w/v) glutaraldehyde for hardening at 30°C. The resulting block was cut into small-size (8 mm) and the gelatin blocks were washed thoroughly with sterile distilled water for complete removal of excess glutaraldehyde [16].

In Polyurethane Foam: Polyurethane foam was cut into equal size cubes (2 cm^3) and sterilized by autoclaving at 121°C for 15 min. After that the cubes were suspended in the cell suspension for 5 min. The cubes were separated from cell suspension and washed 4 times with sterile distilled water [17].

In Scotch Brite: Scotch brite was cut into equal size cubes (2 cm^3) and sterilized by autoclaving at 121°C for 15 min. After that the cubes were suspended in the cell suspension for 5 min. The cubes were separated from cell suspension and washed four times with sterile distilled water [17].

Production of Cellulase Enzymes and Saccharification of Sugarcane Bagasse by Batch Process with Immobilized Cells: The immobilized beads or blocks were transferred into 100 ml of production medium in 250 ml Erlenmeyer flasks. The composition of the production medium was as follows (g/l): Pretreated sugar cane bagasse-80g; Galactose-1g; Malt extract-0.5g; Ammonium molybdate-0.5g; Tween-20-0.2%; NaCl-2.5%; $CaCl_2$-0.2g; pH-6. The flasks were incubated at 37°C for 72 hrs. After incubation, samples were withdrawn for estimation of reducing sugar and assayed for cellulase activity [18].

Production of Cellulase Enzyme and Saccharification of Sugarcane Bagasse by Repeated Batch Process with Immobilized Cells: The reusability of various immobilized cell matrices of *Bacillus pumilus* EWBCM1 was examined. After 72 hrs of incubation, the spent medium was replaced with fresh production medium and the process was repeated up to four batches. The enzyme activity and reducing sugars were estimated for each single batch of fermentation [19].

Analytical Methods: Endo glucanase activity was assayed according to the method of Wood and Baht [20], using carboxy methyl cellulose (CMC) as the substrate. The culture medium was centrifuged at 5000 rpm for 20 min and the supernatant was used as the crude enzyme source. Crude enzyme (0.5 ml) was taken in a test tube with 0.5 ml 1% CMC solution was prepared in 0.2 M citrate phosphate buffer (pH 7) and incubated at 45°C for 30 min. The reaction was terminated by addition of 2 ml of DNS reagent and the tubes were kept at boiling water bath for 5 min. After cooling at room temperature, 7 ml of distilled water was added in each tube and intensity of the colour was read at 540 nm. One unit of enzyme activity was defined as the amount of enzyme required for release 1µ mol of glucose per minute under standard assay conditions. The total reducing sugars was estimated by DNS method [21] and concentration of sugars was estimated from the standard curve of glucose.

Statistical Analysis: The data were statistically analyzed using, two-way analysis of variance (ANOVA) to assess the effect of immobilization of *Bacillus pumilus* EWBCM1 in various matrices on cellulase production and saccharification process [22].

RESULTS AND DISCUSSION

In the present study cellulase enzymes producing bacterium *Bacillus pumilus* EWBCM1 was obtained from Microbiology Laboratory, Ayya Nadar Janaki Ammal College, Sivakasi in Virdhunagar district, Tamil Nadu, India. Several microorganisms including both bacteria and fungi have been found to produce a cellulase enzymes for the degradation of cellulosic materials. These microorganisms can be aerobic and anerobic, mesophilic or thermophilic [23]. Some bacterial species like *Bacillus subtilis, B. macerans, B. pumilus, B.megaterium and Pseudomonas fragi* were used for the production of high amounts of cellulase enzymes through the lignocellulosic waste materials utilizeted. Enzymatic hydrolysis is a natural and ideal method for conversion of cellulose materials to sugars which could be used as a source of food, fuel or chemicals [24].

In the present study the effect of *Bacillus pumilus* EWBCM1 cells immobilized in various matrices, such as calcium alginate, gelatin, agar-agar, polyacrylamide, scotch brite and polyurethane foam for the production of cellulase enzyme. Fig. 1 showed Scotch brite immobilized cells were of high cellulase productivity (0.603 ± 0.03 IU/ml) followed by polyurethane foam (0.566 ± 0.02 IU/ml) and calcium alginate (0.314 ± 0.03 IU/ml). Scotch brite is superior support for immobilization compared to other matrices, where porous structure of the matrix allowed growth of cells inside the pores and higher enzyme productivity [17].

Similar reports were reported by Mukesh *et al* [25] on whole *B. pumilus* strain MK001 cell immobilized of on inert supports for xylanase production. Scotch brite and polyurethane foam matrix immobilized cells were found to be superior in maximizing xylanase production (up to 4000 IU ml^{-1}) using agricultural residues comparing to other matrices studied. Similar reports were reported by Adinarayana *et al* [15] on the low level of alkaline protease production, which was affected with polyacrylamide, gelatin and agar-agar matrices and this may be due to both gluteraldehyde (used for cross linking with gelatin) and polyachrylamide monomers,which are toxic for the cells. The cell leakage form the agar matrix was gradually increased with the increase in fermentation time [26].

Saccharification of sugar cane bagasse showed that, the maximum reducing sugar released (1.63 ± 0.04 mg/ml) by scotch brite immobilized cells of *Bacillus pumilus* EWBCM1 was significant ($p<0.05$) compared with the other matrices (Fig.2). In saccharification process, immobilized cells of *Bacillus pumilus* EWBCM1 applied for efficient conversion of cellulosic substrate into soluble sugars. It is well known that cellulases are inducible enzymes, which are synthesized by microorganisms during their growth on cellulosic materials [1].

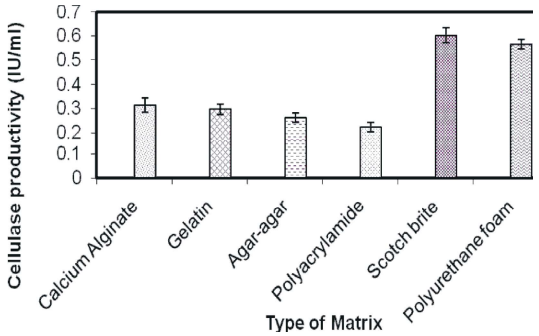

Fig.1: Effect of whole cell immobilization of Bacillus pumilus EWBCM1 on various matrices for the production of cellulase enzyme

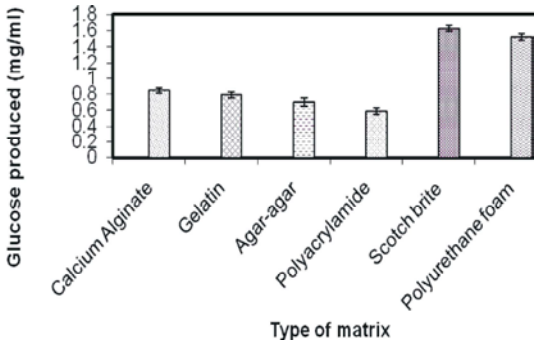

Fig.2: Effect of whole cell immobilization of Bacillus pumilus EWBCM1 on various matrices for saccharification of sugar cane bagasse

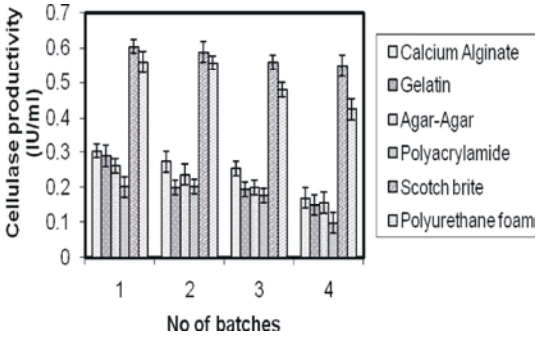

Fig. 3: Production of cellulase enzyme by repeated batch process with immobilized cells of Bacillus pumilus EWBCM1

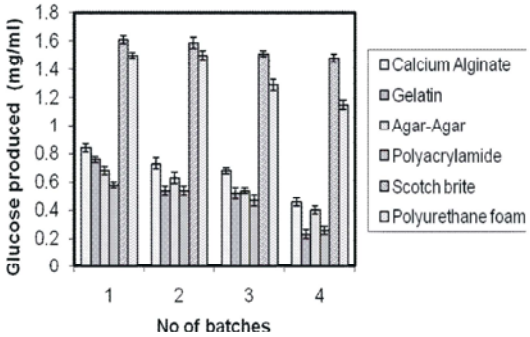

Fig. 4: Saccharification of sugar cane bagasse by repeated batch process with Immobilized cells of Bacillus pumilus EWBCM1

The immobilized *Bacillus pumilus* EWBCM1 cells in various matrices were selected for repeated batch fermentation. Immobilized cells of Scotch brite on used for four batches, it still possessed significant in cellulase production (Fig.3). It has been shown that immobilized cells were able to produce enzyme consistently and that they might be used for continuous enzyme production. In saccharification process, scotch brite immobilized cells was significantly ($p<0.05$) yield high amount of reducing sugar from sugarcane bagasse upto four batches of repeated cycles (Fig.4). The scotch brite matrix was found to be superior to other matrices studied in this paper. In addition, the scotch brite less expensive, non toxic and preparation of biocatalysts involves mild conditions which are added advantage [17].

Similar reports were reported by Mussatto *et al.* [19] the fungus *Aspergillus japonicus* ATCC 20236 was immobilized in scotch brite and used in repeated batch fermentations of sucrose (200 g/l) for the production of beta-fructofuranosidases. The average value of beta-fructofuranosidases activity was a constant 40.6 U/ml at the end of the initial seven cycles. Based on these results, the present immobilization system has a great potential for application in a semi-continuous process for the production of this enzyme at high levels during the overall process.

CONCLUSIONS

Immobilization of *Bacillus pumilus* EWBCM1 cells on scotch brite is a promising method for the cellulase enzyme production. In saccharification process, immobilized cells of *Bacillus pumilus* EWBCM1 used for efficient conversion of cellulosic substrate into soluble sugar was applied in fuel, food and various industrial applications. Specific advantages of cell immobilization technique such as long life-term stability, reusability and reducing the cost of enzyme production.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Ayya Nadar Janaki Ammal College, Sivakasi for providing facility to carry out this work.

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