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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 9 (10): 1923-1928, 2006
ISSN 1028-8880

Production of Acetone, Butanol and Ethanol (ABE) by *Clostridium saccharoperbutylacetonicum* N1-4 with Different Immobilization Systems

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Abstract: The objective of this study is to investigate the production of Acetone, Butanol and Ethanol (ABE) by immobilized cells of *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) under anaerobic batch culture system. Two different methods of immobilization, active immobilization in alginate and passive immobilization by employing stainless steel scrubber, nylon scrubber, polyurethane with uniform pore's size, polyurethane with different pore's size and palm oil empty fruit bunch fiber were studied. Immobilization in alginate was carried out on the effect of cell's age, initial culture pH and temperature on the production of ABE. Immobilized solventogenic cells (18 h) produced the highest total solvents concentration compared to the other phases with productivity of 0.325 g L^{-1} h^{-1}. The highest solvents production by active immobilization of cells was obtained at pH 6.0 with 30°C with productivity of 0.336 g L^{-1} h^{-1}. Polyurethane with different pore's size is significantly better than other materials tested with production of solvents productivity and $Y_{P/S}$ at 3.2 times and 1.9 times, respectively compared to free cells after 24 h fermentation. We conclude that passive immobilization technique increases the productivity (215.12 %) and $Y_{P/S}$ (88.37 %) of solvents by *C. saccharoperbutylacetonicum* N1-4.

Key words: *C. saccharoperbutylacetonicum* N1-4, acetone-butanol-ethanol (ABE), batch culture, passive and active immobilization

INTRODUCTION

During fermentation, Clostridia species produces three major classes of product: solvent (acetone, butanol and ethanol), organic acids (acetate and butyrate) and gases (carbon dioxide and hydrogen) (Datta and Zeikus, 1985). ABE fermentation for butanol production is gaining interest over the petrochemical route (Jones and Woods, 1986). One to the escalating costs associated with chemicals produced synthetically and from fossil resources. Butanol is the most promising solvent compared to acetone and ethanol due to its higher price, better fuel extender than ethanol, low vapour pressure, low miscibility with water and it is completely miscible with diesel fuel even at low temperatures (Qureshi and Blaschek, 2001). Hence the choice of *C. saccharoperbutylacetonicum* in this studies because it is hyper-butanol producing strain and it is resistant to the product inhibition so it can produce a higher concentrations of both butanol and acetone as reported by Ogata *et al.* (1982). Search for high solvent productivity system leads to immobilized cells system (Qureshi *et al.*, 1988). In industrial operation, immobilized microbial cell system could provide additional advantages over freely suspended cells such as facilitating the separation of the cells from the product, high cell densities per reactor volume, high cell concentrations and allowing smaller reactor volumes and greater productivity (Jones and Woods, 1986). Earlier study showed that *C. saccharoperbutylacetonicum* N1-4 cells immobilized in alginate produced higher ABE compared with free cells (Kalil *et al.*, 2003). There is possibility of using other methods of immobilization such as by employing polyurethane foam, nylon, stainless steel scrubber and empty fruit bunch fiber. Alginate is the example as the matrix for immobilization of cells via the entrapment technique, while the materials used for attachment procedure have been synthetic foams like polyurethane (Couto *et al.*, 2004), nylon, stainless steel scrubber and palm oil empty fruit bunch fiber. The objective of the present work was to evaluate production of ABE by *C. saccharoperbutylacetonicum* N1-4 with different immobilization systems. This study was conducted in the Laboratory of Fermentation Technology, Faculty of Engineering, Universiti Kebangsaan Malaysia in year, 2004.

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MATERIALS AND METHODS

Microorganism: *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) was provided by Prof. Dr Yoshino Sadazo from the Laboratory of Applied Microbiology, Department of Agricultural Chemistry, Kyushu University and the culture stock was maintain in 15% potato glucose media (PG medium: 150 g mesh potato, 10 g D-glucose, 0.5 g $(NH_4)_2SO_4$ and 3 g $CaCO_3$). One milliliter of culture stock was transferred into 9 mL PG media and heat-shocked in boiling water for 1 min. Then cultured at 30°C for 24 h and used as an inoculum.

Media: TYA medium employed contained the following compounds per liter of distilled water: 40 g D-glucose, 2 g yeast extract, 6 g tryptone, 3 g CH_3COONH_4, 10 mg $FeSO_4.7H_2O$, 0.5 g KH_2PO_4 dan 0.3 g $MgSO_4.7H_2O$. The initial pH was adjusted to 6.5 with 1M NaOH and sterilized at 121°C for 15 min.

Alginate entrapment method: Immobilization of N1-4 cells in alginate was done (Kalil *et al*., 2003), using cells from three different growth phases (acidogenic, solventogenic and sporogenic). The acidogenic, solventogenic and sporogenic cells were obtained by growing the bacterium for 10, 18, 24 and 72 h, respectively.

Support materials: Five packing materials were used throughout this study, stainless steel scrubber (average width 0.5 mm), nylon scrubber (2.5×2.3× 0.4 cm in cubes), polyurethane with uniform pore's size with average diameter 200-400 μm (2.5×2.3×1.2 cm in cubes), polyurethane with different pore's size with average diameter 20-900 μm (2.5×2.3×1.2 cm in cubes) and palm oil empty Fruit Bunch Fiber (EFB) (average width 1 mm). All the packing material was packed at the bottom of the test tube and sterilized at 121°C for 15 min.

EFB as alternative substrate: Two sets of medium with the entrapment of EFB were prepared to test whether there is a possibility that the EFB fiber component can act as carbon sources for the production of solvents. The mediums were TYA medium and TYA without glucose.

Fermentation: Static batch fermentations were carried out in this study. One hundred milliliter test tube with 30 mL working volume were packed with 1 g of cuttings stainless steel scrubber, three cubes of nylon scrubber, two cubes of polyurethane with uniform pore's size, two cubes of polyurethane with different pore's size and 2 g EFB fiber were then inoculated with 10% (v/v) inoculum and incubated at 30°C for 120 h. Two test tubes will be taken out from water bath for analyzing sample at different intervals.

Analytical determinations: Suspended cell biomass concentration was estimated as Dry Cell Weight (DCW) by measuring the optical density at 660 nm using spectrophotometer (UV-1201V, Shimadzu Corporation, Japan) and relating it between the optical density and DCW. The concentration of acetone, butanol, ethanol and butyrate was determined with gas chromatography (HP5890, Hewlett Packard), equipped with a capillary column Hp-InnoWax (30 m × 0.25 mm id × 0.25 μm). Helium was used as the carrier gas at a flow rate of 13 mL min. The temperature of Flame Ionization Detector (FID) was 280°C and the temperature of injector was 220°C. Acetate produced during fermentation was measured using HPLC (Agilent 1100 Series, Agilent Technologies). The separation of acids was detected at wavelength 120 nm using column C18 and 7 mM H_2SO_4 as mobile phase with flow rate at 0.6 mL min^{-1}. The column temperature was kept constant at 40°C. The consumption of glucose was estimated by determination of reducing sugars by DNS method using D-glucose as a standard. The polyurethane pore's size was estimated by reverse phase-contras microscope (Nikon Eclipse TS100, Japan) with 4×10 magnification.

RESULTS AND DISCUSSION

ABE fermentation with immobilized cells using alginate: ABE fermentation by free cells of *C. saccharoperbutylacetonicum* N1-4 gives the value of productivity (0.262 g L^{-1} j^{-1}) and $Y_{P/S}$ (0.492 g solvents g^{-1} $glucose^{-1}$) at 72 h of fermentation with total

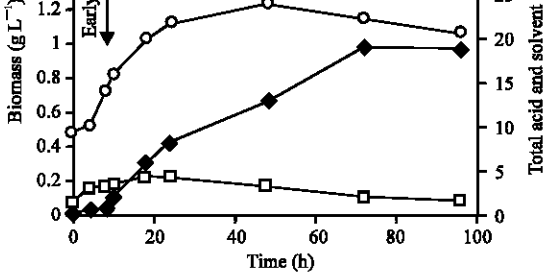

Fig. 1: Profile of solvents production and cell growth at 30°C in TYA

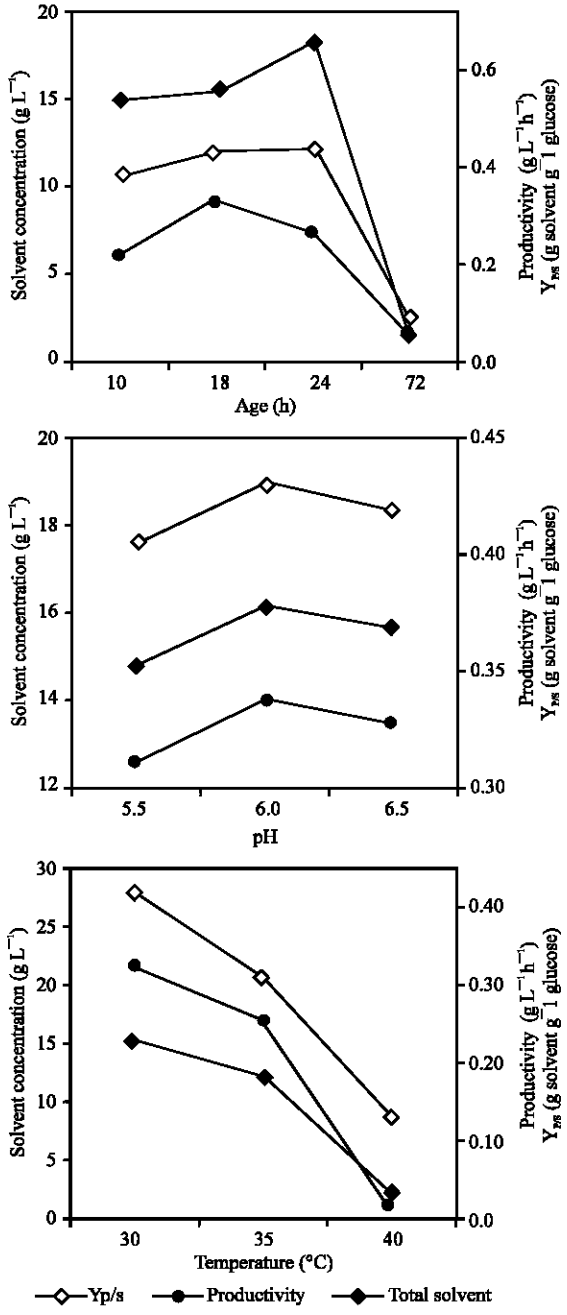

Fig. 2: Effect of cell's age, pH and temperature on the concentration of total solvent, productivity and $Y_{P/S}$

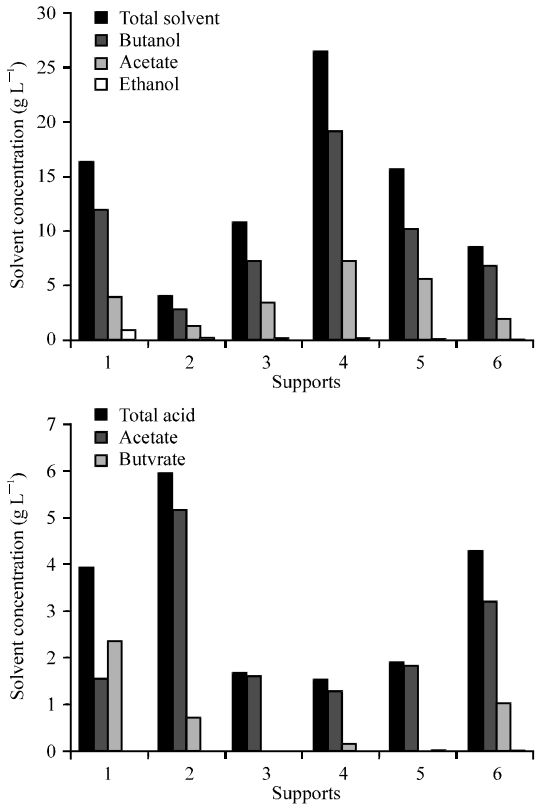

Fig. 3: The concentration of solvent and acid after 24 h of ABE fermentation at 30°C in TYA. (1; stainless steel scrubber 2; nylon scrubber 3; polyurethane with uniform pore's size 4; polyurethane with different pore's size 5; empty fruit bunch fiber 6; free cells)

solvent concentration of 19.12 g L^{-1} (Fig. 1). However the highest productivity was obtained at 24 h fermentation. Active immobilization experiments using alginate utilizing cells from three different growth phases (acidogenic, solventogenic and sporogenic) was to overcome the problem of inhomogeneous and containing cells with different in morphology and physiology or mature spore in culture (Jones and Woods, 1986). Figure 1 shows that, cells were harvested at four cell's age and were concentrated 1.5 times before immobilization in alginate beads. First harvest was from growing cells at 10 h and less solvent was produced at this time (early). Another two variations were at the point when growth nearly ceased at 18 and 24 h of cultivation. At this time, cell density nearly reached the maximum and solvent production was in progress ('middle'). The last harvest was at 72 h when solvent production showed no increase and biomass concentration started to drop are primarily due to cell lysis (late).

Effect of cell's age, initial pH and temperature: From Fig. 2 shows that, immobilized solventogenic cells at 18 h produced the highest total solvents concentration compared to at 24 h with productivity of 0.325 g $L^{-1}h^{-1}$ at 48 h fermentation. The productivity at 48 h fermentation appeared to be obtained by

skipping out from log growth phase because gel particle of 1.5 times concentrated solventogenic (18 h) immobilized cells was used. This result are in agreement with Kalil *et al.* (2003) who reported that ABE production could be improved when high concentrations of cells at solventogenic growth phase was used. Further studies utilizing immobilized solventogenic cells (18 h) to examine the effect of initial pH (pH 5.5, 6.0) and temperature (35, 40°C) to increase the solvents production, resulted in the highest solvents production obtained at pH 6.0. Madihah (2002) also reported initial pH of 6.0 for solvent fermentation. Present result showed that, the lowest productivity and $Y_{P/S}$ of ABE was obtained at initial pH 5.5 after 48 h fermentation. That lower solvent production might be due to this initial pH used was not an optimal pH to support the growth of N1-4 strain in the beads (Bahl *et al.*, 1982; Monot *et al.*, 1984). So anaerobic bacteria could not reach high cell population in the immobilized cell gel particle.

The effect of temperature showed that ABE productivity and $Y_{P/S}$ decreases with the increase in temperature (Fig. 2). However, fermentation with 35°C could induce higher acetone production while temperature of 30°C would enhance butanol production. Schoutens *et al.* (1985) also reported that in batch cultures a temperature of 30°C was found to be more favorable for the butanol production than 37°C. Besides that, the $Y_{P/S}$ obtained at 30°C was higher about 33.76% than fermentation at 35°C (Fig. 2). These results are in agreement with that reported by Yu and Fang (2003).

From the study of active immobilization using alginate, productivity of solvent by immobilized solventogenic cells (pH 6, 30°C) with 1.5 times concentrated cells resulted in 1.3 times higher productivity than free N1-4 cells in TYA with butanol concentration at 13.081 g L^{-1}. However, the overall productivity achieved by free cells was 26% higher than immobilized cells. This decreased in overall productivity are due to more times needed in immobilization procedure and cells not being in contact with the substrate due to floating beads. Therefore, it can be concluded that the entrapment technique is not a suitable immobilization technique for high solvent production. Passive immobilization technique seems to offer a better approach.

ABE fermentation with passive immobilization using five packing materials: The immobilization by attachment on five packing materials was carried out to investigate the effect on solvent concentration, productivity and yield ($Y_{P/S}$) in TYA medium at 30°C. Batch fermentations for the production of ABE by freely suspended cells at 24 h fermentation was compared by cells immobilized in packing materials. Immobilized cells on polyurethane with different pore's size produced the highest solvent concentration (26.679 g L^{-1}) on the 24th h of cultivation with 19.228 g L^{-1} butanol and 7.450 g L^{-1} acetone (Fig. 3). Immobilized cells on cuttings of stainless steel scrubber produced the second highest total solvent concentration of about 16.243 g L^{-1}, followed by 15.650 g L^{-1} when empty fruit bunch fiber was used, 10.606 g L^{-1} in polyurethane matrix with uniform pore's size and 3.863 g L^{-1} in nylon scrubber matrix after 24 h of fermentation (Fig. 3). Ethanol production occurred in fermentation using stainless steel scrubber at a concentration of 0.743 g L^{-1} with ABE ratio of 5:16:1 (Fig. 3). The result support the report by Montoya *et al.* (2000) that high butanol production usually seems to be coupled with low ethanol production.

The total acid concentration with polyurethane with different pore's size was the lowest compared to other packing materials and free cells with 1.373 g L^{-1} acetate and 0.235 g L^{-1} butyrate. Mostly, it was found that the lower total acid concentration achieved was in the higher solvent production of immobilized system. Packing system with stainless steel scrubber produced the second highest acid and solvent concentrations. From this observation, we can conclude that different packing material would have different effect in producing acid or solvent. The highest concentration of acid (6.139 g L^{-1}) was recorded with nylon scrubber packing. This may be due to acids produced become accumulated in the nylon matrix. Interaction of acids-nylon is a possible explanation.

EFB as alternative substrate: The support material can be inert or biologically active (Shuler and Kargi, 1992), a good support material should be rigid and chemically inert. EFB as packing material used in the experiment consists of carbon source that could assist in the production of solvents. Therefore the doubtful result obtained from the experiment should be count. Fermentation with EFB in TYA medium without glucose, could produce 3.237 g L^{-1} of solvents after 24 h. So, we know that about 15.71% extra of solvents counted from 0 h was produced by the fermentation using EFB itself as carbon source.

Results showed that solvent productivities achieved by using different packing materials were 1.084 g L^{-1} h^{-1} for polyurethane with different pore's size, 0.648 g L^{-1} h^{-1} for stainless steel scrubber, 0.633 g L^{-1} h^{-1} for empty fruit bunch fiber, 0.406 g L^{-1} h^{-1} for polyurethane with uniform pore's size and 0.122 g L^{-1} h^{-1} for nylon scrubber

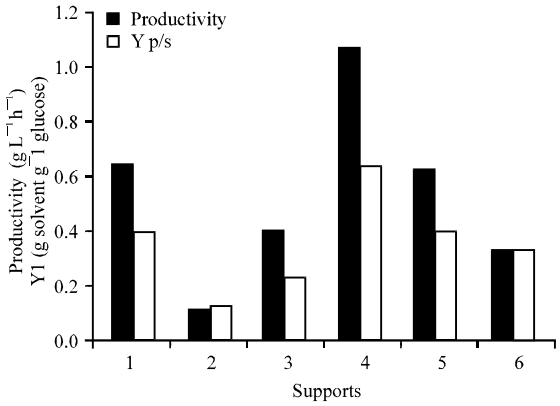

Fig. 4: Productivity and yield ($Y_{P/S}$) after 24 h of ABE fermentation at 30°C in TYA. (1; stainless steel scrubber 2; nylon scrubber 3; polyurethane with uniform pore's size 4; polyurethane with different pore's size 5; empty fruit bunch fiber 6; free cells)

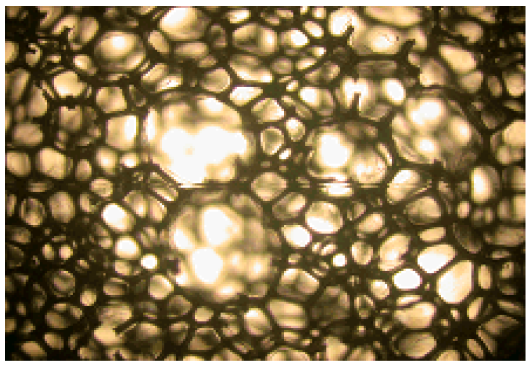

Fig. 5: Polyurethane with different pore's size

(Fig. 4). The lowest production of solvent achieved by immobilized system with nylon scrubber was similar to the result obtained by Cauto *et al.* (2004). They reported that nylon scrubber as packing material gave the lowest laccase activity by *T. hirsuta* in dye decolorization.

We conclude that, the attachment technique with cells immobilized in polyurethane with different pore's size gave very good result. Polyurethane with different pore's size is the best packing materials because it could increase the solvents productivity and $Y_{P/S}$ by 3.2 times and 1.9 times, respectively compared to free cells. Polyurethane with different pore's size has the properties of a good support material for entrapments because of it's porous and consist of large number of very small pore sizes of about 20-900 μm (Fig. 5). Most microorganisms tend to attach firmly on surfaces by an adhesive polymer produced by the cells and polyurethane with different pore's size is a suitable support material. Polyurethanes are well known for their ability to entrap or immobilize biological materials. O'Reilly and Crawford (1989) have successfully used polyurethane as immobilization matrices for living microbial cells in biodegradation of toxic chemicals. Besides that, Monahar *et al.* (2001) also reported that the degradation of naphthalene by *Pseudomonas* sp. immobilized in the polyurethane foam. Polyurethanes has other advantages over other packing materials in that is cheap support material and readily available. Besides that, the process is simple, fast and no other chemical is required for cell immobilization and thus contributing to high productivity of solvents especially butanol with A:B ratio is 1: 2.6 and it is suggested to be used in scaled up system of ABE fermentation by *C. saccharoperbutylacetonicum* N1-4.

ACKNOWLEDGMENTS

The authors would like to thank Yayasan Felda for their financial support through a grant D/9/99 and Prof. Dr Yoshino Sadazo of Kyushu University, Japan for supplying *C. saccharoperbutylacetonicum* N1-4.

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