Screening of PHA Producing *E. coli* from Municipal Sludge Waste and Influence of Phosphate on PHA Production

K. Mamatha[1] P. Murali[2] K. Suresh[3] N. Mathiyazhagan[4]
[1,2,3,4]MGR College, Hosur, Krishnagiri, Tamilnadu, India

Abstract— In the present study, an attempt was made to isolate efficient PHA producing bacteria from municipality sludge soil sources. The obtained *E. coli* isolates have the accumulation of polyhydroxyalkanoates (PHA) within their cells. The PHA producing colonies were given dark blue colored colonies as positive for polyhydroxyalkanoates production and secondarily confirmed by light orange color colonies on selective media shows positive for polyhydroxyalkanoates. The amount of PHA accumulated in *E. coli* indicating a growth-related production where the final amount of PHA obtained depended on maximum biomass produced. The maximum PHA production (27.1mg/L) was recorded at 96 h cultivation in the medium using 8% phosphate in *E. coli* as co-nutritional source. The intensity of the methylene band near 2966 cm^{-1}, provided additional information for PHA characterization in the FTIR analysis. In NMR analysis, 2-5mg of the copolymer sample is dissolved in deuteriated chloroform (CD Cl3) (1.0ml) and high resolution 'H-NMR spectrum is recorded on a NMR spectrometer. The ^1H NMR spectra of the samples and the standard are almost identical, conferring that extracted intracellular compounds are polyhydroxyalkanoates (PHAs). Further the presence of phosphate could directly serve as an inexpensive nutrient source for production of biodegradable plastic PHA.

Keywords: *E. coli*, polyhydroxyalkanoates, phosphate, FTIR, NMR analysis

I. INTRODUCTION

The entire biodegradable polymer is defined as a biopolymer that is completely renewed by living organisms, usually microorganisms, to carbon dioxide, water and humic material by utilizing various organic sources such as sugarcane, potato starch or the cellulose from trees, straw and cotton (Mahishi et al. 2003). Biodegradable materials under development include polyglycolic acids, polyhydroxyalkanoates (PHAs), and polylactides (Kim, et al., 2009). Amongst these, PHAs are of particular interest because they possess thermoplastic characteristics and resemble synthetic polymers to a larger extent. Plastics produced from PHAs have been reported to be truly biodegradable in both aerobic and anaerobic environments further more than 80 different forms of PHAs have been isolated from bacteria such as Azotobacter, Bacillus, Archaebacteria, Methylobacteria, Pseudomonas have been found to synthesize PHA to varying levels. However the accumulation of PHA is not a prerequisite, they are stated to support the sporulation process in bacillus spp. PHA, as a reduced compound, acted as a sink for excess electrons, especially in nitrogen fixing bacteria by providing protection to the oxygen-labile nitrogenase (Sudesh, et al., 2011).

The fabrication of biodegradable plastics on a large scale is limited because of the relative expense of the substrate and low yield of polymer. According to Li, et al., (2007), the higher production costs, especially raw material costs, make it quite difficult for PHA to compete with conventional petroleum-based plastics in the commercial market place. Hence, alternative strategies for PHA production are being investigated so researchers. PHA production costs could be reduced by several means by using cheaper substrates such as starch, whey and enhancement of product yield, by using recombinant *E. coli* (Yang, 2010). There have been some investigations on the possibility of producing PHA in transgenic plants from few decades onwards (Nawrath et al. 1994). PHAs are natural thermoplastic polyesters, which can be used for manufacture of disposable items such as razors, utensils and different personal hygiene products. They can be used in the manufacture of latex paints (Steinbuchel and Hein, 2001). PHAs also promise to be a new source of small molecules, some of which have potential applications as biodegradable solvents. These include β-hydroxy acids, 2-alkenoic acids, β-hydroxyalkanols, β-acyllactones, β-amino acids, and β-hydroxyacid esters (Sodian et al., 2000).

This biodegradable and biocompatible PHAs have applications in medical therapeutics, further it can be used to fabricate three-dimensional, porous, biodegradable heart valve scaffold bone fracture fixation, manufacture of surgical pins, sutures, staples, swabs, fixation rods and cardiovascular stents (Scholz, 2000). PHAs can be used as carriers for long term slow release of drugs, insecticides, herbicides and fertilizers and in wound dressing. In view of the countless threads of traditional plastics and its usages, biodegradable plastics are straightway required to decrease the adverse worldwide economic and environmental effects.

II. MATERIALS AND METHODS

A. Collection and Processing of Soil Sample

The sludge waste soil samples were collected from the municipality of Hosur taluk, Krishnagiri district of Tamilnadu. The collected samples were immediately processed and refrigerated for further process in the PG and Research Centre in Biotechnology of MGR College, Hosur.

B. Isolation of PHA producing predominant bacteria from municipal waste soil

The PHA producing bacteria was isolated by the methods of Khardenavis et al. (2003) with slight modifications. Standard serial dilution protocol was followed and serially diluted samples were inoculated on freshly prepared sterile nutrient agar plate by perform in spread plate method and labeled properly and incubated at 28°C for 48 hrs in incubator.

C. Screening of PHA Producing Bacteria

All the bacterial isolates (10nos) were qualitatively tested for PHA production following the viable colony method using Sudan Black B dye (Juan et al., 1998). For rapid screening of PHA producers, nutrient agar medium was supplemented with 1% of sterilized glucose. The plates were incubated at 30 0C for 24 hours. After the incubation 0.02% of Sudan

Black B stain was spread over the colonies and kept undisturbed for 30 minutes. The excess stain from the colonies were washed with ethanol. The dark blue colored colonies were taken as positive for PHA production (Juan et al., 1998).

D. Fluorescent Staining Method

The PHA producing isolates were confirmed by fluorescent staining method using acridine orange, as suggested by Senthilkumar and Prabhakaran (2006). Ten µl of 48 hr old culture of the isolate was transferred to an eppendorf tube containing 50 µl of acridine orange ("Himedia") and incubated for 30 minutes at 300C. After the incubation, the culture was centrifuged at 4000 rpm, for 5 min. The pellet was collected and suspended in distilled water. A smear was prepared on a clean microscopic slide and observed under microscope. The presence of yellow colored granules inside the cell indicate PHA component.

E. Characterization of PHA Producing Bacterial Isolates

Among ten isolates, the most predominant and effective PHA producing bacteria was elected and were subjected to a set of morphological, physiological, standard biochemical tests and different staining techniques (grams and spore staining) and selective media for the purpose of identification. After the identification of PHA producing bacteria from these biochemical tests, the subculture was prepared and maintained in refrigerator for further use.

F. Preparation of culture medium and Fermentation for PHB production

All the PHA producing isolates were subjected to submerged fermentation in Minimum mineral medium (M medium), containing (per liter) $Na_2HPO_4.2H_2O$ 4.5g; KH_2PO_4 1.5g; $MgSO_4.7H_2O$ 0.2g; $NaCl_2$ 0.9g; $(NH_4)2SO_4$ 2g; $CaCl_2$ 0.02g; NH_4 Fe citrate 0.05g; trace element solution SL6 1 ml; Glucose 20g (Chien, et al., 2012). In this sterile Minimum mineral media the selected bacteria was inoculated and incubate for 72 hrs for quantification test.

G. Extraction Assay for PHA

Bacterial cells containing polymer were collected after centrifugation at 4000 rpm for 10 min .Then pellet was suspended in equal volume of 4% sodium hyphochlorite and incubated at 37°c for 24 hour. Pellet was washed with acetone, ethanol and water to remove the unwanted materials. The whole mixture was centrifuged again and the supernatant was discarded. Finally obtained polymer granules were dissolved in hot chloroform (Arkin et al., 2000).

H. Analysis of PHA-Fourier transform- infrared Spectroscopy (FT-IR)

The extracted PHA was subjected for IR spectra recording in the range 4000-400 cm^{-1} at a resolution of 4 cm^{-1} to confirm the functional groups of the polymer as per the method described by Liu, et al., (2014). Melting point of the polymer was estimated to examine the polymer stability at higher temperature (BUCHI B-545, Switzerland). All the experiments were performed in triplicates and all the data is represented here is the mean+/-SD. The presence of different functional groups in PHA was checked by FTIR. Extracted PHA (2 mg) was dissolved in 500 µl of chloroform and layered on $Nacl_2$ crystal. After evaporation of chloroform, PHA polymer film was subjected to FTIR.

I. NMR Spectroscopy

The individual monomer unit was confirmed by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. ^{1}H-NMR spectra of PHA sample were recorded in CDC13 on Bruker ACF 300 spectrophotometer at 300 MHz by using "Tetramethylsilane" as internal standard. The presence of different functional groups in PHA was checked by NMR Spectroscopy. Extracted PHA was dissolved in DMSO solution .PHA polymer was subjected to NMR Spectroscopy.

III. RESULTS

The competent isolates were selected and their culture parameters were analyzed and conformed as *E. coli* based on their morphological, growth on selective media and biochemical results.

A. Rapid screening of isolates for PHA production by plate assay method:

The bacterial colonies were examined for PHA accumulation by staining with Sudan Black (0.3 g in 70%ethanol) by using rapid screening method. The dark blue colored bacterial isolates were grown on nutrient agar medium supplemented with 1% glucose, it considered as positive for polyhydroxyalkanoates production and light orange color colonies were observed on selective media it confirms the polyhydroxyalkanoates (Figure 1 & 2).

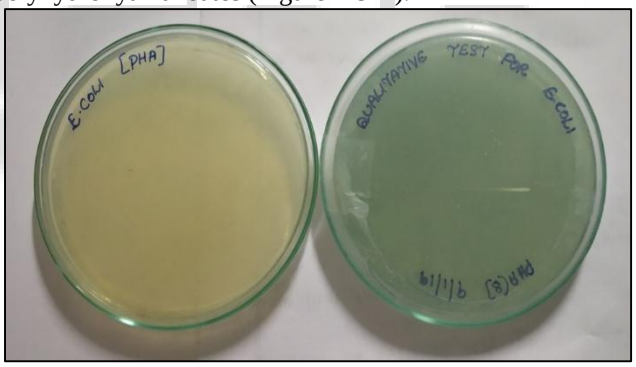

Fig. 1: Sudan block B staining method

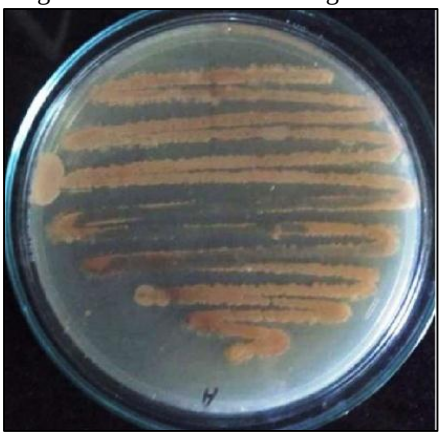

Fig. 2: PHA producing *E. coli* on PHA selective media

B. Fluorescent Staining Method

Primary detection of PHA production in the isolates were confirmed by sudan black staining method, again it was also

confirmed by fluorescent staining method using acridine orange, the yellow colored granules were observed inside the cell indicate the development of PHA (Figure 3).

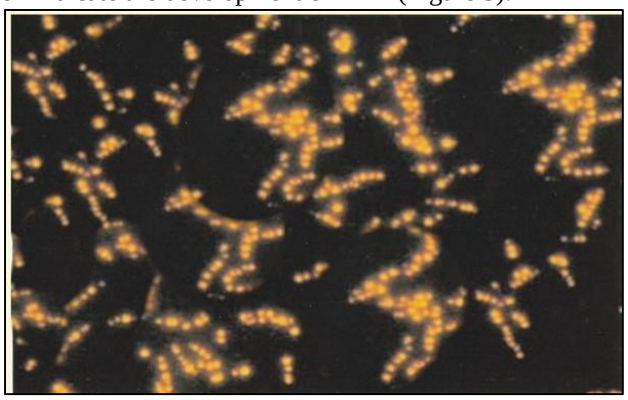

Fig. 3: Acridine orange stained *E. coli* under a fluorescent microscope.

C. Quantification of PHA production by Spectrophotometry

The concentration of the PHA in the isolated bacteria was suspended in chloroform and sulfuric acid was used as blank the result were determined by spectrophotometry at 400nm, and optical density value was recorded as 1.189484. The amount of PHA accumulated in *E. coli* indicating a growth-related production where the final amount of PHA obtained depended on maximum biomass produced. The maximum PHA production of *E. coli* was 27.1mg/L at 96 h cultivation in the medium using 8% phosphate as co-nutritional source (Figure 4).

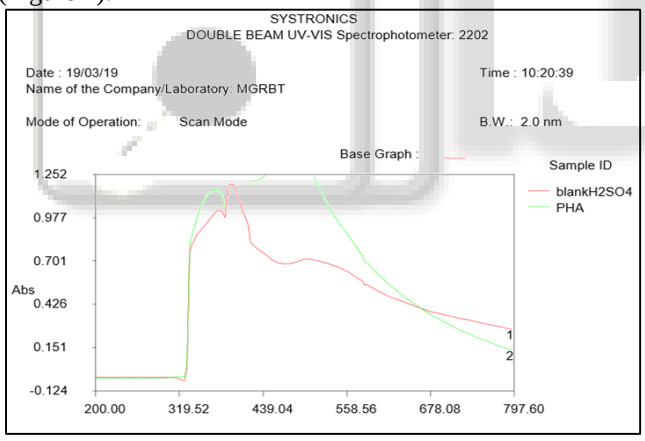

Fig. 4: Quantitative analysis of PHA from *E. coli*

D. Extraction of PHA and FTIR Analysis

Crud paste like polymer granules were harvested from *E. coli* and it was confirmed by FTIP analysis. The FTIR spectra of pure PHA comprising short chain length monomers, such as hydroxybutyrate (HB), monomers including hydroxyoctaoate (HO), medium chain length polyhydroxy alkanoates (mclHA), and hydroxydecanoate (HD), and mcl HA monomers showed their strong characteristic band at 1631cm^{-1}, 1548 cm^{-1}, 1409 cm^{-1} respectively. The intensity of the methylene band was near 2966 cm^{-1}, it provided additional information for PHA characterization and confirmation. (Figure 5).

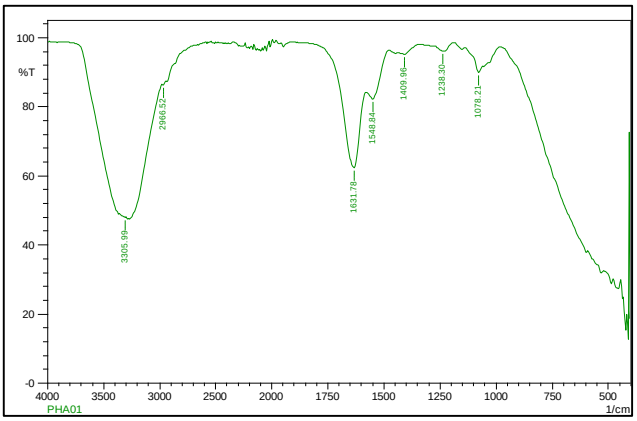

Fig. 5: FT-IR analysis of PHA produced by E.coli

E. NMR analysis of PHA

The composition of the PHA elements in a copolymer was determined by analyzing the nuclear magnetic resonace (NMR) spectra. In NMR analysis, 2-5mg of the copolymer sample was used. The results showed the the structures of polyesters of *E. coli* was showed that the following resonance signals like: HC=CH bond at 3.363 ppm, CH_2O–COOH bond at 2.548 ppm, $_1$H NMR spectra (Figure 10) of the PHAs extracted from *E. coli* (Figure 6). The ^{1}H NMR spectra of the samples and the standard are almost identical, conferring that extracted intracellular compounds from *E. coli* were polyhydroxybutyrates (PHAs).

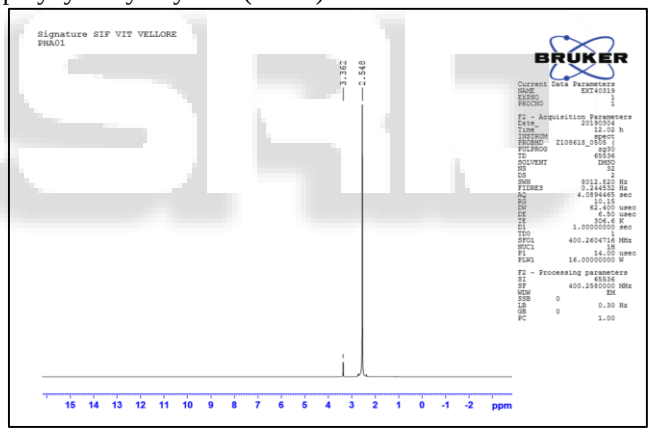

Fig. 6: NMR analysis of PHA produced by E.coli

IV. Discussion

The major concerns about the commercialization of PHAs is the high production costs compared with traditional plastic materials (Yee, et al., 2003). A strategy for identifying and isolation of suitable PHAs-producing micro-organisms from the environment is possible. In this study several strains obtained from sewage sludge belong to *E. coli* which was effectively produced PHA. Qiu et al., (2006) were also reported that PHA produced *E. coli* derived from soil sample. Similarly Aeromonas hydrophila and many species and strains of Pseudomonas have been reported to produce fine quantity of PHAs (Qiu et al., 2006). In this study the additional nutritional source of media with phosphate shows positive impact on PHA production (Saranya and Shenbagarathai 2011). This might be due to the metabolic stimulating effect of phosphate on PHA production. As evident, ammonium sulphate was the best supporter for the

growth and PHA production by B. subtilis as it increased the cell dry mass and PHA production up to 36.98 and 22.98 g/L, respectively. However, in case of *E. coli*, ammonium nitrate supported the highest growth and PHA production, which were 26.76 and 15.70 g/L, respectively. Sheu, et al., (2000) reported that the presence of inorganic chemicals such as ammonium salts as a source of nitrogen is a significant need during the growth phase in order to maximize the concentration of biomass responsible for the accumulation of PHA. Verlinden et al., (2007) has reported that PHA production is enhanced under limiting conditions of nitrogen and phosphorus. In the present study, the positive effect of increased concentrations of nitrogen sources on the growth and PHA production by *E. coli* was studied by the addition of various concentrations (0.5 – 8.0 g/L) of phosphate in the form of dihydrogen phosphate. Phosphate at a concentration of 8 g/L supported the PHA production, which was 27.1mg for *E. coli*. Beyond these concentrations, PHA production by the two strains decreased drastically.

Phosphate was an important factor for PHA production. Ryu et al., (1997) adopted phosphate limitation strategy to induce PHA accumulation. However, phosphate was also required for the cell growth. On this study, the maximum PHA production by *E. coli* (23.11 and 15.74 g/L, respectively) was recorded in the presence of 2 g/L of KH_2PO_4 and $Na_2HPO_4.2H_2$. Beyond these concentrations, both the growth and PHA production decreased drastically by the two strains.

As far the effect of other mineral salts, MgSO4 and CaCl2 had no effect either on cellular growth, or PHA concentration by *E. coli*. The results were in agreement with those of Srikanth pilla (2011) who reported that MgSO4.7H2O (1- 10 mm) and trace elements solution (1-20 ml/L) did not affect PHA production in mineral medium.

V. Conclusion

The *E. coli* isolated from Hosur Municipality sludge was able to utilize the phosphate as co-nutrient source for PHA production. The first line of impression of this study concludes that the phosphate could directly serve as an inexpensive nutrient source for production of biodegradable PHA. Thus, this study may solve the problem of high production cost of bioplastic and help in the reduction of traditional plastic which were used in the commercial production.

Acknowledgment

The authors would like to thank the department of Biotechnology, M.G.R. College, Hosur for providing laboratory support for successful completion of this research work.

References

[1] Arkin, A. H. Hazer B. and Borcakli, M., (2000). Chlorination of poly (3-Hydroxyalkanoates) containing unsaturated side chains. Macromolecules. 33: 3219-3223.
[2] Chien CC, Li HH, Soo PC, Chen SY, and Wei YH,. (2012). Effects of different substrate composition on biosynthesis of polyhydroxybutyrate-cohydroxyvalerate by recombinant Escherichia coli. Appl. Biochem. Biotechnol. 166: 796-804.
[3] Juan, M. L., Gonzalez, l. W. and walker, G. C., (1998). A novel screening method for isolating exopolysaccharide deficient mutants. Appl.Environ. Microbio. 64: 4600-4602.
[4] Khardenavis, A., Sureshkumar and Chakrabarti, J., (2003). Biodegradable plastics Production from industrial waste water by activated sludge. In : 44th annual conference of association of microbologists of India. 107.
[5] Kim do Y, Park DS, Kwon SB, Chung MG and Bae KS, (2009). Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolyesters with a high molar fraction of 3-hydroxyvalerate by an insect-symbiotic Burkholderia sp. IS-01. J Microbiol. 47: 651-656.
[6] Li R, Zhang H, Qi Q (2007). The production of polyhydroxyalkanoates in recombinant Escherichia coli. Bioresour. Technol. 98: 2313-2320.
[7] Liu, F., Li, W., Ridgway, D. and Gu, T., (2014). Production of poly-hydroxybutyrate on molasses by recombinant Escherichia coli. Biotech let. 20: 345-348.
[8] Mahishi, L. H., Tripathi, G. and Rawal, S. K., (2003). Poly--hydroxybutyrate (PHB) Synthesis by recombinant Escherichia coli harbouring, streptomyces aureoficiens phb biosynthesis genes: effects of various carbon and nitrogen sources. Microbio. Res. 158 : 19-27.
[9] Nawrath, C., Prior, Y. and Somerville, C., (1994). Targeting of the poly- hydroxybutyric acid biosynthetic pathway to the plastids of arabidopsis thaliana results in high levels of polymer accumulation. Proceedings of national academic science, 91: 12760-12764.
[10] Qiu, Y.Z., Han, J. and Chen, G.Q. (2006). Metabolic engineering of Aeromonas hydrophila for the enhanced production of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate). Appl. Microbio. Biotech. 69: 537–542.
[11] Ryu, H. W., Hahn, S. K., Chang, Y. K. and Chang, H. N., (1997). Production of poly (3-Hydroxybutyrate) by high cell density fed-batch culture of Alcaligenes eutrophus with phosphate limitation. Biotech. Bioengi. 55: 27-32.
[12] Saranya V. and Shenbagarathai R. (2011). Production and characterization of PHA from recombinant E.coil harbouring PHAC1 gene of indigenous Pseudomonas sp. LDC-5 using molasses. Brazilian J. Microbio. 42. 1109-1118.
[13] Scholz, C., (2000). Poly-hydroxyalkanoates) as potential biochemical material: an overview. In : (ed.) Scholz, C., gross, r. A. Polymers from renewable resources biopolymers and biocatalysis. Acs series, 764: 328-334.
[14] Senthil kumar, B. and Prabhakaran, G., (2006). Production of PHB (bio plastics) using bio-effluents as substrates by Alcaligens eutropha. Indian J. Biotech. 5: 76-79.
[15] Sheu, D. S., wang, Y. T. and Lee, C.Y., (2000). Rapid detection of polyhydroxyalkanoate accumulating bacteria isolated from the environment by colony pcr. Microbiol. 146: 2019-2025.

[16] Sodian, R., Sperling, J. S., Martin, D. P., Egozy, A., Stoc, K. U., Mayer, J. E. and Vacnti, J. P., (2000). Fabrication of a trileaflet heart value scaffold from a polyhydroxy alkanoate biopolyester for use in tissue enigeering. Tissue Engi. 6: 183-188.
[17] Srikanth Pilla (2011). Handbook of Bioplastics and Biocomposites Enginerring Applications.Massachusetts : Wiley –Scrivener publishing LLC.
[18] Steinbuchel, A. and Hein, S., (2001). Biochemical and molecular basis of polyhydroxyalkanoic acids in microorganisms. Adva. Bioche. Engi. Biotech. 71: 81-122.
[19] Sudesh K, Bhubalan K, Chuah JA, Kek YK, and Kamilah H, (2011) Synthesis of polyhydroxyalkanoate from palm oil and some new applications. Appl. Microbiol. Biotechnol. 89: 1373-1386.
[20] Verlinden RA, Hill DJ, Kenward MA, Williams CD, and Radecka I., (2007). Bacterial synthesis of biodegradable polyhydroxyalkanoates. J. Appl. Microbiol. 102: 1437-1449.
[21] Yang YH, Brigham CJ, Budde CF, Boccazzi P, and Willis LB, (2010). Optimization of growth media components for polyhydroxyalkanoate (PHA) production from organic acids by Ralstonia eutropha. Appl. Microbiol. Biotechnol. 87: 2037-2045.
[22] Yee PL, Hassan MA, Shirai Y, Wakisaka M, Abdul Karim MI (2003). Continuous production of organic acids from palm oil mill effluent with sludge recycle by the freezing-thawing method. J. Chem. Engi.Japan 36: 707-710.

