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Biotechnological Conversion of Wastewater to Polyhydroxyalkanoate by *Bacillus* in a Sequencing Batch Reactor

Kannika Chookietwattana and N. Khonsarn

Faculty of Technology, Mahasarakham University, Mahasarakham, Thailand

Abstract: In this paper, *Bacillus megaterium* A9an was selected as an efficient *Bacillus*-producing polyhydroxyalkanoate (PHA) on the basis of intracellular PHA detection by the nile red staining method and PHA determination by spectrophotometry. A sequencing batch reactor (SBR) cycle of 24 h was fed with a synthetic wastewater and seeded with a pure culture of the selected *Bacillus*-producing PHA to generate an activated sludge. Two anoxic/aerobic times of 4 h/18 h and 2 h/20 h were used to determine the most suitable operational condition for conversion of wastewater to PHA. The results showed that the PHA production, the total Kjeldahl nitrogen (TKN) and orthophosphate removal efficiencies were higher at the longer anoxic time while the COD removal efficiency was higher at the shorter anoxic time. Under anoxic/aerobic times of 4 h/18 h, the highest average PHA production and yield of 1.4054 g l^{-1} and 74.0% as dry sludge weight was found. The highest average COD, TKN and orthophosphate removal efficiencies were 87.5%, 80.0% and 57.2%, respectively. Thus, the use of *B. megaterium* A9an in SBR operating at the anoxic/aerobic times of 4 h/18 h could offer a promising approach for PHA production along with a high performance in wastewater treatment.

Key words: Polyhydroxyalkanoate · PHA · Polyhydroxybutyrate · Bioplastic · *Bacillus* · Sequencing batch reactor

INTRODUCTION

Nowadays, petroleum-based plastics pollution and the scarcity of fossil fuels have encouraged research towards the development of microbially-produced polymers, polyhydroxyalkanoate (PHA) [1]. PHA is polyesters of hydroxyalkanoate which has properties similar to synthetic plastics and are completely biodegradable in the environment [2]. PHA is synthesized by numerous bacteria as a storage compound for carbon and energy when a carbon source is excessive and the nutrients (nitrogen, phosphorus, sulfur or oxygen), which are essential for growth, are present in limited concentrations [3-5].

The commercial PHA production is only by the Gram-negative bacteria because of higher productivity [4,6]. It seems that the *Bacillus* was neglected as a potential PHA producer because the accumulated PHA could be utilized during the sporulation stage [7, 8]. However, PHA produced by the Gram-negative bacteria contains lipopolysaccharide (LPS) endotoxins which are pyrogenic in human beings [9, 10] and thus provide a chance for *Bacillus* as a potential PHA producer. Aside from the problem concerning co-purification of the endotoxin with PHA produced by the Gram-negative bacteria, another limit to bioplastic usage is the high PHA production cost which is raised by the supplementary carbon source. The carbon source is the main expense for the raw materials and raises PHA production costs to nine times that of synthetic plastic production [11]. Thus, the usage of wastewater as a carbon source to feed wastewater treatment plant and harvest the activated sludge for further PHA extraction is an attractive approach to reduce the PHA production cost. This approach also reduces the waste activated sludge to be managed and is therefore reduce the operation cost of wastewater treatment [12].

During this decade, several attempts were made to produce PHA by the *Bacillus* [8, 13, 10, 14-18]. PHA production which was achieved by some *Bacillus* strains isolated from activated sludge, have been reported elsewhere [19-21] and all works were carried out in term

Corresponding Author: Kannika Chookietwattana, Department of Biotechnology, Faculty of Technology, Mahasarakham University, Kantarawichai District, Mahasarakham, 44150, Thailand. Tel: +66-4375-4333.

of fermentation studies. On the other hand, a lot of work has been done on PHA production by activated sludge in wastewater treatment systems which acetate was added to stimulate growth of phosphate accumulating organisms in the system [22-26]. However, little information is available on the use of *Bacillus* as a seed for PHA production by activated sludge. Thus, the objectives of the present research are selecting an efficient *Bacillus*-producing PHA, determining the potential of the selected bacterium in production of PHA and treating wastewater in an anoxic/aerobic sequencing batch reactor (SBR) system without supplementation of acetate to the synthetic wastewater.

MATERIALS AND METHODS

Microorganism and Culture Media: Fifteen *Bacillus* strains, namely: *B. aquimaris* A9ax, *B. bataviensis* A7aA, *B. firmus* A8ak, *B. flexus* A9s, *B. licheniformis* A8aT, *B. megaterium* AIIy, *B. megaterium* A5aG, *B. megaterium* A12ag, *B. megaterium* A9an, *B. mycoides* A9ay, *B. pumilus* Apau1, *B. subtilis* A9aDD, *B. vallismortis* A1az, *B. vietnamensis* A1C, *B. vietnamensis* A8e, isolated from saline soil in Thailand, were used in this study. They were identified using phenotypic characterization, chemical analysis and genotypic characterization (unpublished data in Chookietwattana, K. 2003. Ph.D. Thesis). E2 medium was used for PHA production [27]. The medium contains 3.5 g l^{-1} $NaNH_4HPO_4.4H_20$, 7.5 g l^{-1} $K_2HPO_4.3H_20$, 3.7 g l^{-1} KH_2PO_4, 10 ml l^{-1} of 100 mM $MgSO_4.7H_20$ and 1 ml l^{-1} of MT microelements stock solution (2.78 g of $FeSO_4.7H_20$, 1.98 g of $MnCl_2.4H_20$, 2.81 g of $CoS0_4.7H_20$, 1.47 g of $CaCl_2.2H_20$, 0.17 g of $CuCl_2.2H_20$ and 0.29 g of $ZnSO_4.7H_20$ per one liter of 1 N HCl).

Chemical and Reagents: Nile red and poly-β-hydroxybutyrate (PHB) were purchased from Sigma (USA). All other chemicals and solvents of analytical grade were obtained from other commercial suppliers.

Selection of *Bacillus*-Producing PHA: All *Bacillus* strains were singly cultivated on E2 agar and incubated at 28°C. After incubation for 48 h, bacterial cells from the grown colonies of each strain were smeared and stained with 0.1 mg ml^{-1} of nile red (dissolved in acetone) [28] to detect the presence of PHA granules. Only two maximum PHA producers were selected based on the emitted fluorescence intensity which was observed using an Olympus BX60 fluorescence microscope (Olympus, Japan). They were then grown in 50 ml of E2 broth and incubated at 28°C in a shaker at 150 rpm. After incubation of 48 h, cell suspension (10 ml) was centrifuged at 10,000 rpm for 10 min. The cell pellets were washed twice with 10 ml sterile normal saline and then subjected for PHA observation by a transmission electron microscope and PHA determination.

Transmission Electron Microscopy (TEM): The cell pellets obtained were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer solution for 2 h at 4°C. The suspension was centrifuged at 2,000 rpm for 10 min. The pellets were rinsed in phosphate buffer and post-fixed with 1% OsO_4 in distilled water for 2 h. The cells were then washed three times in 0.1 M phosphate buffer solution (pH 7.2) each for 10 min and stained with 4% uranyl acetate. After staining for 2 h at room temperature, the cells were dehydrated through a graded series of acetone (20, 40, 60, 80, 100 and 100% v/v) each for 10 min. Specimens were then infiltrated with acetone mixes (acetone:epon) of 2:1 for an hour and 1:1 for an hour, followed by a 1:2 mix overnight. These specimens were then penetrated with pure epon for 3 h. The samples were embedded by capsule beam followed by polymerization at 60°C for 24 h. Thin sections of 60-90 nm were cut and stained in lead citrate and photographed with a transmission electron microscope JEM 1230 (JEOL, Japan) in the Central Laboratory, Faculty of Science, Mahasarakham University, Thailand.

Inoculum Preparation: The selected bacterium was inoculated to two flasks containing 250 ml of tryptic soy broth [29]. The flasks were incubated at 30°C for 24 h in a shaker (New Brunswick Scientific Model C24, USA.) at 200 rpm. An inoculum was obtained by centrifugation of bacterial suspension at 8000 rpm for 10 min, using a Sorvall RC 5C centrifuge, Dupont, U.S.A.

SBR Experiment: The SBR experimental system was composed of a glass reactor equipped with an aerator, agitator and sample line (Fig 1). A 5-l working volume SBR was seeded with the selected bacterial inoculum (1 g of cells wet weight per 1 l of synthetic wastewater). The synthetic wastewater comprised 791.6 mg l^{-1} sucrose, 10 mg l^{-1} urea, 375 mg l^{-1} NH_4HCO_3, 283.3 mg l^{-1} $NaHCO_3$, 20 mg l^{-1} KH_2PO_4, 15.8 mg l^{-1} $MgSO_4•7H_2O$, 2.8 mg l^{-1} $CaCl_2•2H_2O$, 1.4 mg l^{-1} $FeCl_3•6H_2O$. This composition provided the chemical oxygen demand (COD) at 1,000±50 mg l^{-1}. The cycle time of the reactor was operated at 24 h per day through four modes as shown in Fig 2.

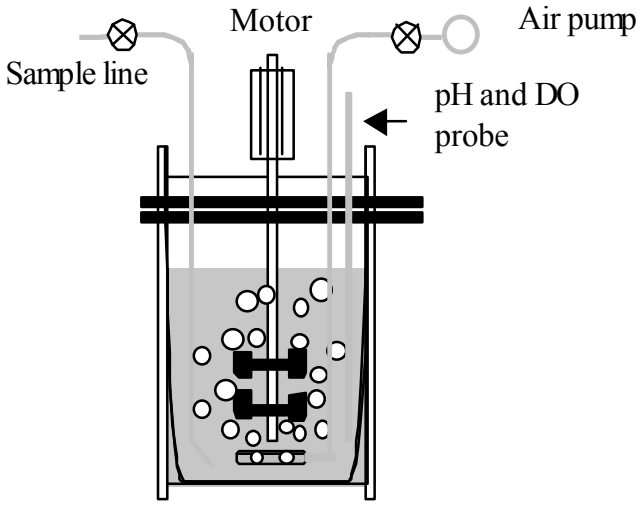

Fig. 1: A schematic diagram of the SBR.

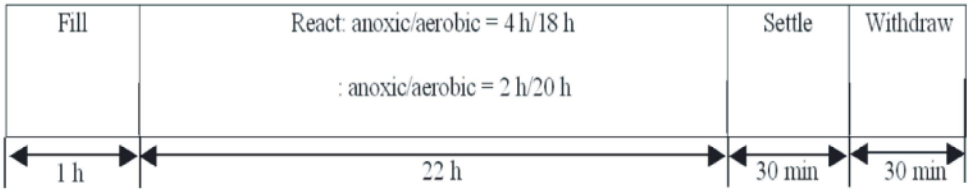

Fig. 2: Operating modes of the 24 h SBR cycle. For providing an anoxic condition, only the mixing of wastewater was operated while the aeration was accompanied in the aerobic condition.

Bacterial cultures were acclimated to the synthetic wastewater and the altering anoxic/aerobic conditions in the react mode. The system was run with the anoxic/aerobic times at 4 h/18 h until the steady state was reached. It was then switched to anoxic/aerobic at 2 h/20 h in order to evaluate the influence of oxygen on the system performance. The pH of the SBR was controlled at the level of 7.0±0.2 by adding HCl or NaOH solution. The dissolved oxygen (DO) was kept below 0.5 mg l^{-1} and above 2 mg l^{-1} during anoxic and aerobic conditions, respectively. In each cycle, before a settling phase was implemented, approximately 200 ml of mixed liquor suspended solids (MLSS) were taken for MLSS measurement. Four liters of effluent were withdrawn at the end of each cycle and filled up with the fresh synthetic wastewater to equilibrate the working volume and to maintain a 1.25-day hydraulic retention time. Steady state conditions were achieved when reactor performance, as measured by COD and MLSS, remained constant and had good sludge settling properties. The system performance data were collected when the system was operated for five consecutive cycles after the steady-state was reached. Effluents and the settled activated sludge were collected from the reactor at the withdrawal period in order to evaluate the system performance in removal of total COD, total Kjeldahl nitrogen (TKN) and orthophosphate and the PHA production in activated sludge.

Analytical Procedure: The total COD, total TKN, orthophosphate and MLSS were analyzed according to the Standard Methods for the Examination of Water and Wastewater [30]. pH and DO were measured by using a pH meter model 511275-AB (Beckman, USA) and a YSI 200 DO meter (YSI Inc., China), respectively. PHA was extracted from the activated sludge using the solvent extraction method as described by Punrattanasin [22]. PHA content was determined through spectrophotometric analysis according to the method described by Yillmaz *et al*. [13]. Due to PHB is the most common forms of PHA in activated sludge, in this study, PHA content was analyzed in term of PHB. The standard curve was prepared by using a serial dilution of pure PHB. Quality control was ensured using standards as well as triplicates and the average values were reported.

RESULTS AND DISCUSSION

Screening of *Bacillus*-Producing PHA: PHA, a lipid-like polymer, is synthesized by PHA-producing bacteria for carbon and energy reserves. They are stored in granular inclusion bodies in the cytoplasm. Various dyes such as sudan black B [31] and nile blue A [32] have been widely used for phenotypic detection of cytoplasmic PHA granules but nile red is a highly sensitive stain among others for this purpose [33-35]. It is thus used in the present study to distinguish between PHA producing and non-producing bacteria. Fluorescence of the cytoplasmic PHA granules of fifteen *Bacillus* strains stained with nile red was investigated under UV-light. All of them exhibited fluorescence. Although several species of the genus *Bacillus* have been reported to be PHA producers [36], our results possessed other *Bacillus*-producing PHA, namely: *B. aquimaris*, *B. bataviensis, B. flexus*, *B. vallismortis* and *B. vietnamensis*, which have never been recognized as PHA producers. Based on the emitted fluorescence intensity, *B. megaterium* A12ag and *B. megaterium* A9an, the first two isolates exhibiting high fluorescence intensity, were chosen for quantification of PHA production. After cultivation in E2 broth at 28°C for 48 h, *B. megaterium* A9an showed the highest dry cell weight and PHA production at 0.9957 g l^{-1} and 0.2191 g l^{-1}, respectively (Fig 3). Aslim *et al*. [37] isolated 27 *Bacillus* spp. from soil from Ankara, Turkey and found that the bacteria produced PHB between 0.040-0.207 gl^{-1} when cultivated in nutrient broth for 48 h. Our results correspond to the study of Aslim *et al*. [37] in which the highest PHB production was found in the *B. megaterium* but PHA production by our *B. megaterium* strain was a negligible higher. Our results also showed a higher PHA production than the study of Yilmaz *et al*. [13] which determined the PHB production by 31 *Bacillus* spp. and found the highest PHB production at only 0.097 g l^{-1} in *B. cereus* M15. The TEM micrograph also confirmed the granular inclusion bodies of PHA produced (Fig 4). Therefore, *B. megaterium* A9an was selected as an efficient *Bacillus*-producing PHA for further study in SBR experiment.

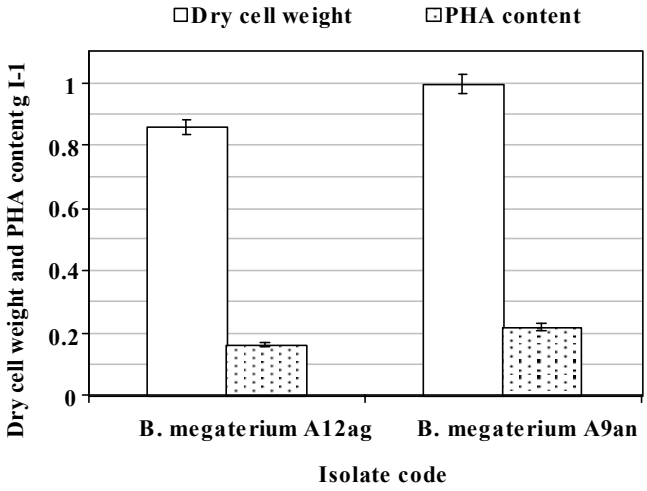

Fig. 3: Cell biomass and PHB production by *B. megaterium* A9an cultivated in E2 broth at 28°C for 48 h. The error bars represent the SD (n=3).

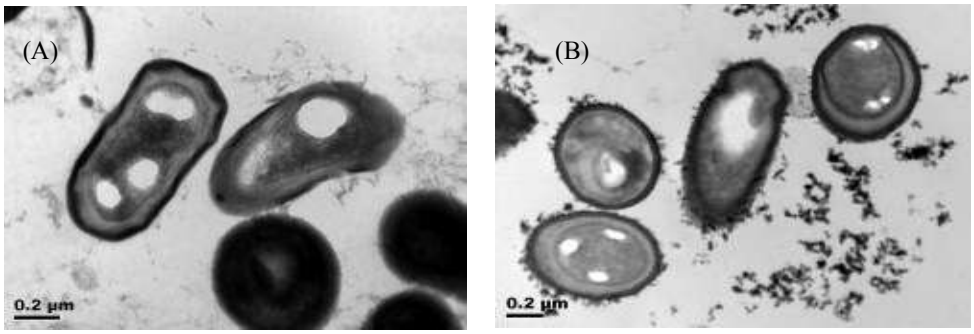

Fig. 4: Visualization of granular inclusion bodies in the cytoplasm of *B. megaterium* A9an (A) and *B. megaterium* A12ag (B) under TEM.

Performance of SBR in Treating Wastewater and PHA Production: The PHA synthesis and the uptake/release of polyphosphate are the most important functions of polyphosphate accumulating organisms (PAO) in enhanced biological phosphorus removal (EBPR) processes [38, 39]. Thus, in EBPR systems, PAO also act as potential PHA-producing organisms. These important findings led to a new challenge approach for PHA production by activated sludge from wastewater treatment systems. An enhancement of phosphorus and nitrogen removals can be accomplished by providing alternative anaerobic/aerobic conditions for microbial population in the system. A SBR system was chosen for PHA production in this study because of the ease in controlling anoxic and aerobic conditions, cost efficiency and enhancement of nutrient removal. The selected *Bacillus*-producing PHA, *B. megaterium* A9an, was inoculated to the SBR reactor and allowed to acclimatize to the synthetic wastewater containing sucrose as a sole carbon source. The system was started-up with the anoxic/aerobic times of 4 h/18 h in the react mode. During the first week of the start-up period, the MLSS concentration (microbial biomass) was substantially decreased due to the adaptation of the bacterial seeding to the synthetic wastewater. Then the activated sludge was accomplished to form a good floc within 22 days and its concentration was varied from 1,504 mg l^{-1} to 2,063 mg l^{-1} (data not shown) which met the guidelines of design parameters for SBR [40]. The start-up period took 26 days to reach the steady state and showed the overall removal efficiency of COD, TKN and orthophosphate at 87.5%, 80.0% and 57.2%, respectively. The reactor also achieved good sludge-settling as the average effluent suspended solid concentration was only at 18.4 mg l^{-1}. Generally, the start-up period of SBR could be reduced by the bioaugmentation of the reactor with a conventional activated sludge. In the study of Sarioglu [41], three types of inocula were used: a mixture of conventional activated sludge and *Acinetobacter lwoffi*; a mixture of conventional activated sludge and *Pseudomonas aeruginosa*; and a mixture of conventional activated sludge, *Pseudomonas aeruginosa* and *Acinetobacter lwoffi*, to start up the anaerobic/aerobic SBR reactors with the aim to enhance phosphorus removal. The first type of inocula mixture used a start-up time of 35 days, while the next two mixtures used 19 days to reach the steady state. When considering the start-up period of this study and the study of Sarioglu [41], the *B. megaterium* A9an was well acclimatized to the alternating anoxic/aerobic conditions, although the conventional activated sludge was not added to the inoculum. In addition, the COD removal efficiency of our anoxic/anaerobic SBR reactor was within the range of typical SBR at 85-95%. When the anoxic/aerobic times of 2 h/20 h were applied, the COD removal efficiency increased to 97.5% with the COD effluent concentration of only 32 mg l^{-1} while the removal efficiency of TKN and orthophosphate decreased to 68.9% and 55.0%, respectively (Fig 5). These results are due to the fact that the growth rate of bacteria in aerobic conditions is higher than anoxic/anaerobic conditions and leads to an increase in the conversion of carbonaceous organic matter to new bacterial cells [40]. In consequence, a better sludge-settling property was obtained and the average effluent suspended solid concentration was reduced to 10.8 mg l^{-1} (data not shown). Unlike the removal of organic matter, nitrogen and phosphorus removals require a more complex series of reactions and a sufficiently long anoxic time in the reactor [42]. Meanwhile, an increase of aerobic time from 18 h to 20 h may also affect the nitrogen removal process, since dissolved oxygen can suppress the enzyme system which is needed for denitrification [43]. Thus, a decrease of anoxic time from 4 h to 2 h resulted in depletion of TKN removal efficiency. Nevertheless, TKN removal efficiency under anoxic/anaerobic times of 2 h/20 h was still within the range of the nitrogen removal performance of a conventional activated sludge system [40]. The influence of a shortened anoxic time on phosphorus removal was negligible. This result could be due to the PAO in the system that could degrade polyphosphate causing the release of orthophosphate in consequent under both aerobic and anoxic conditions [26].

As shown in Fig 6, the activated sludge of SBR inoculated with the selected *Bacillus*-producing PHA, *B. megaterium* A9an, was clearly accumulated PHA. PHA production and yield under anoxic/aerobic times of 4/18 h were higher than at 2/20 h. Under anoxic/aerobic times of 4 h/18 h, the activated sludge with an average MLSS concentration of 1.9047 g l^{-1} provided the average PHA content and yield of 1.4054 g l^{-1} and 74.0% as dry sludge weight, respectively. With respect to MLSS concentration, the activated sludge obtained from the anoxic/aerobic times of 2 h/20 h showed a much higher growth than those obtained from the anoxic/aerobic times of 4 h/18 h, nevertheless, the average PHA content and yield were lower. These results could have been due to a limited oxygen concentration is a preferred condition for PHA synthesis and accumulation [39] while an increasing of oxygen concentration results in bacterial cell proliferation. Satoh *et al*. [12] found that the anaerobic

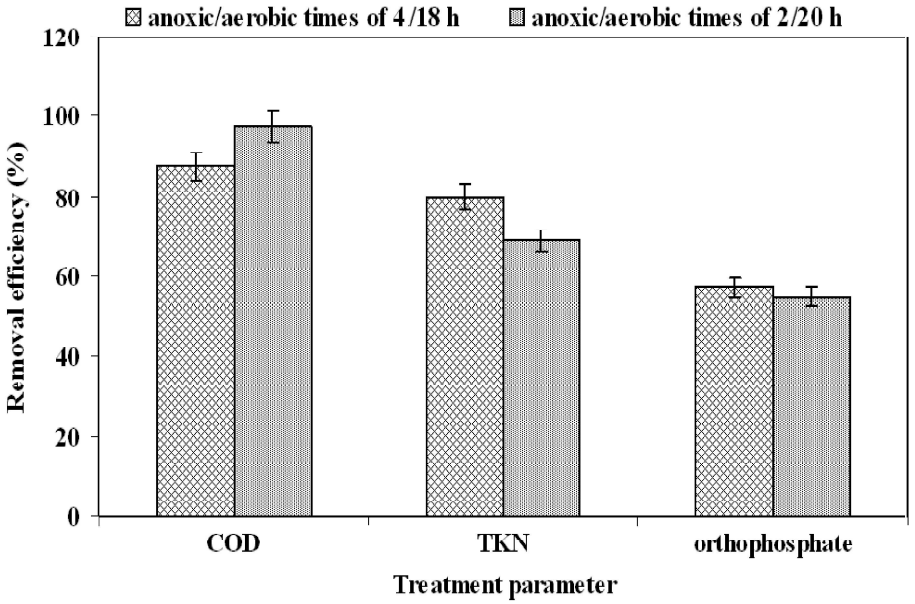

Fig. 5: Treatment performance of SBR system seeded with the *B. megaterium* A9an at different conditions of reaction phase. The error bars represent the SD (n=3).

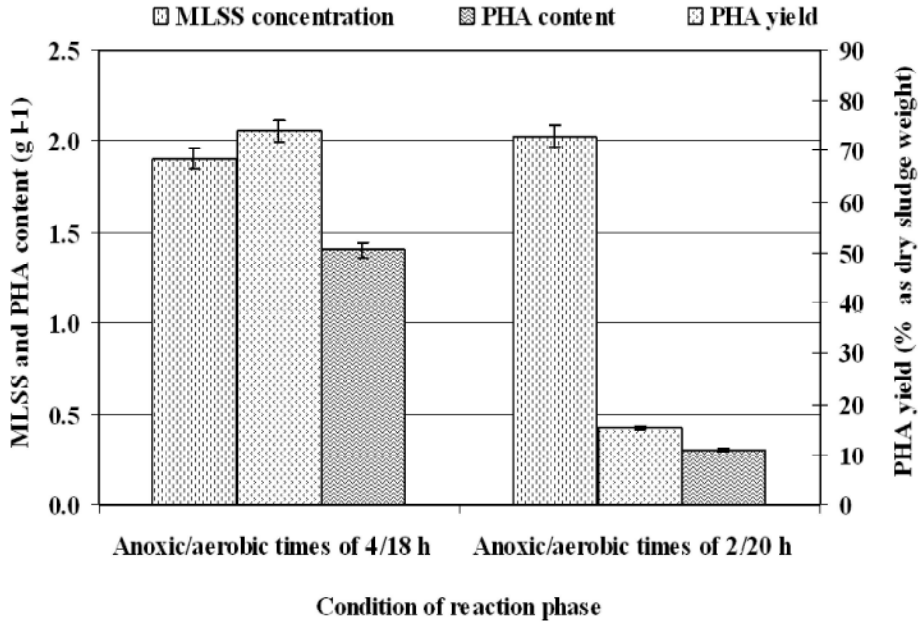

Fig. 6: The biomass and PHA production by activated sludge produced from the SBR system seeded with the *B. megaterium* A9an at different conditions of reaction phase. The error bars represent the SD (n=3).

SBR accumulated PHA of 33% as dry sludge weight and the PHA accumulation was increased up to 62% as dry sludge weight in a microaerophilic/aerobic SBR. A similar result was also found in the study of Punrattanasin [22]. On the other hand, Takabatake *et al*. [44] used the aerobic batch reactors to produce PHA by activated sludge samples which were obtained from four wastewater treatment plants in Tokyo, Japan. The highest PHA accumulation in the activated sludge was only 29.5% of dry cell weight. Rodgers and Wu [26] reported that anaerobic/aerobic conditions provided higher PHB production than either the anaerobic or aerobic condition alone. Our results therefore coincided with the other previous studies [12, 22, 42, 26] in which an anoxic condition benefits PHA accumulation in the activated sludge.

Concerning carbon source for PHA production by activated sludge, organic acids especially acetate were used as a sole carbon source for PHA production in most of the work [22-26]. The sludge which was produced from an anaerobic/aerobic SBR fed with municipal wastewater (COD 60-130 mg l^{-1}) containing 0-20 mg l^{-1} of acetate, accumulated PHA at 30% as dry sludge weight [23]. In order to enhance PHB production, Kumar *et al*. [17] determined an optimal concentration of acetic acid to enrich growth of activated sludge which was obtained from a food processing wastewater treatment plant. The activated sludge was enriched in a synthetic medium containing acetic acid (500 mg l^{-1} to 3,000 mg l^{-1}). The maximum biomass concentration of 3,150 mg l^{-1} was obtained at 2,000 mg l^{-1} of acetic acid within 96 h of incubation. The maximum PHA yield was 33% of biomass (w w^{-1}). Rodgers and Wu [26] achieved PHA production of 50% when acetate concentration of 750 mg l^{-1} was applied to the anaerobic/aerobic SBR. A positive effect of acetate supplementation on PHA production by *Cupriavidus necator* DSMZ 545, cultivated in a mineral medium containing sugarcane molasses under batch conditions, was also reported [45]. Dave *et al*. [46] investigated the PHB production by petrochemical activated sludge under nitrogen and phosphorus limited and carbon excess conditions. They found that various bacterial species inhabited in the sludge but the sludge that accumulated high PHB was dominated by *Bacillus* sp. They concluded that the sludge containing *Bacillus* sp. provide a high potential for PHB production. Thirumala *et al.* [47] has also isolated some *Bacillus* spp. producing PHB from activated sludge. Therefore, the PHA contents obtained in this study was higher than the other investigations dealing with PHA production by SBR system which only acetate was supplemented to the influent [22-26]. A high PHA content obtained could be due to a bioaugmentation of *B. megaterium* A9an, although sucrose was used as a sole carbon source. The ability of *B. megaterium* A9an to grow and accumulate PHA in medium devoid of acetate could offer an advantage for PHA production by reducing the carbon source expense.

A comparison of PHA production by fermentation process using the *Bacillus* spp. reveals that the PHA yields were varied among species within a range of 11%-69% as dry cell weight. PHB production of 35% as dry cell weight was produced by the *Bacillus* sp. JMa5 in a fed-batch fermentation which molasses containing 50 g l^{-1} of sucrose was used as a carbon source [8]. The dry cell biomass of 70 g l^{-1} was reached after 30 h of fermentation. Valappil *et al.* [14] carried out a large-scale PHA production from *B. cereus* SPV in a 20 l fermentor (fed-batch experiments) which glucose concentration was maintained above 10 g l^{-1}. The PHB content of 38% as dry cell weight was obtained within 48 h with the biomass concentration of 3.0 g l^{-1}. Kulpreecha *et al*. [16] reported the highest biomass of 72.5 g l^{-1} and PHB content of 42% as dry cell weight from fed-batch cultivation of *B. megaterium* BA-019 in a minimal salt medium. *Bacillus mycoides* RLJ B-017 which was isolated from an activated sludge provided the highest biomass concentration and PHB yield of 3.6 g l^{-1} and 69.4% as dry cell weight, when it was grown in a medium containing 20 g l^{-1} sucrose and 2.0 g l^{-1} di-ammonium sulphate [48]. To date, *Bacillus* sp. 87I [21] which was isolated from soil of Hyderabad, India, is the most efficient PHA producer among the *Bacillus* spp. reported to be the PHA producers. It yielded a maximum PHB of 70.04% as dry cell weight in glucose containing medium. Although, PHA production from the fermentation systems using pure cultures provides much higher cell biomass than the wastewater treatment system, the production process requires sterilization procedures and complicated processes for separation of biomass from the fermentation broth which then raises the production cost. To compete with PHA production by the fermentation systems, PHA production by activated sludge in wastewater treatment system should employ inoculation of efficient PHA producers which can store high PHA content and grow rapidly in wastewater without supplementation of an expensive substrate because the biomass concentration in the wastewater treatment system is (normally not exceed 5,000 mg l^{-1}) much lower than those obtained from the fermentation systems. According to the results reported above, an application of an efficient *Bacillus*-producing PHA, *B. megaterium* A9an, to the SBR system could enhance PHA accumulation of the activated sludge to the level which is comparable to the highest reported so far by the other *Bacillus* spp.

CONCLUSION

In the present work, a biotechnological method for production of PHA by wastewater activated sludge in a SBR system which inoculated with the selected *Bacillus*-producing PHA strain, *Bacillus megaterium*

A9an, is proposed. The anoxic/aerobic times of 4 h/18 h are the most suitable condition for reaction phase of a 24 h SBR aiming for PHA production. The anoxic/aerobic times of 2 h/20 h offer a better performance in COD removal but nutrient removals and PHA content were lower than the previous condition. The *B. megaterium* A9an is a promising PHA producer strain for production of PHA in a SBR system which also functions for wastewater treatment because it efficiently grows and produces high PHA in the wastewater without supplementation of acetate. As a consequence of our findings we believe that the cost of PHA production using only wastewater and the bioaugmentation of *B. megaterium* A9an is a less costly alternative to the other methods which require the additional cost of acetate to stimulate growth of PAO in the SBR system. However, effects of carbon sources, shorter cycle times, pH and carbon to nitrogen ratio require further study in order to maximize and accelerate the PHA production which could then provide sufficient information for process development of an industrial scale production.

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REFERENCES

1. Khanna, S. and A.K. Srivastava, 2005. Recent advances in microbial polyhydroxyalkanoates. Process Biochem., 40: 607-619.
2. Reddy, C.S.K., R. Ghai, Rashmi and V.C. Kalia, 2003. Polyhydroxyalkanoates: an overview. Bioresour. Technol., 87: 137-146.
3. Anderson, A.J. and E.A. Dawes, 1990. Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev., 54: 450-472.
4. Lee, S.Y., 1996. Bacterial Polyhydroxyalkanoates. Biotechnol. Bioeng., 49: 1-14.
5. Steinbüchel, A. and B. Füchtenbusch, 1998. Bacterial and other biological systems for polyester production. Trends Biotechnol., 16: 419-427.
6. Chen, G.Q., 2005. Industrial Production of PHA. In: G.-Q. Chen (ed). Plastics from Bacteria: Natural Functions and Applications. Springer-Verlag, New York, pp: 121-131.
7. Slepecky, R.A. and J.H. Law, 1960. Synthesis and degradation of poly-β-hydroxybutyric acid in connection with sporulation of *Bacillus megaterium.* J. Bacteriol., 82: 37-42.
8. Wu, Q., H. Huang, G. Hu, J. Chen, K.P. Ho and G.Q. Chen, 2001. Production of poly-3-hydroxybutyrate by *Bacillus* sp. JMa5 cultivated in molasses media. Antonie van Leeuwenhoek., 80: 111-118.
9. Lee, S.Y., J.I. Choi, K. Han and J.Y. Song, 1999. Removal of endotoxin during purification of poly (3-hydroxybutyrate) from Gram-negative bacteria. Appl. Environ. Microbiol., 65: 2762-2764.
10. Valappil, S.P., A.R. Boccaccini, C. Bucke and I. Roy, 2007. Polyhydroxyalkanoates in Gram-positive bacteria: insights from the genera *Bacillus* and *Streptomyces*. Antonie van Leeuwenhoek., 91: 1-17.
11. Serafim, L.S., P.C. Lemos, R.F. Oliveira and M.A.M Reis, 2004. Optimisation of polyhydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions. Biotech. Bioeng., 87: 145-160.
12. Satoh, H., I. Iwamoto, T. Mino and T. Matsuo, 1998. Activated sludge as a possible source of biodegradable plastic. Water Sci Tech., 38: 103-9.
13. Yilmaz, M., H. Soran and Y. Beyatli, 2005. Determination of poly-*b*-hydroxybutyrate (PHB) production by some *Bacillus* spp. World J. Microbiol. Biotechnol., 21: 565-566.
14. Valappil, S.P., S.K. Misra, A.R. Boccaccini, T. Keshavarz, C. Bucke and I. Roy, 2007. Large-scale production and efficient recovery of PHB with desirable material properties, from the newly characterized *Bacillus cereus* SPV. J. Biotechnol., 132: 251-258.
15. Halami, P.M., 2008. Production of polyhydroxyalkanoate from starch by the native isolate *Bacillus cereus* CFR06. World J. Microbiol. Biotechnol., 24: 805-812.
16. Kulpreecha, S., A. Boonruangthavorn, B. Meksiriporn and N. Thongchul, 2009. Inexpensive fed-batch cultivation for high poly (3-hydroxybutyrate) production by a new isolate of *Bacillus megaterium*. J. Biosci. Bioeng., 107(3): 240-245.
17. Kumar, T., M. Singh, H.J. Purohit and V.C. Kalia, 2009. Potential of *Bacillus* sp. to produce polyhydroxybutyrate from biowaste. J. Appl. Microbiol., 106: 2017-2023.

18. Pandian, S.R., V. Deepak, K. Kalishwaralal, N. Rameshkumar, M. Jeyaraj and S. Gurunathan, 2010. Optimization and fed-batch production of PHB utilizing dairy waste and sea water as nutrient sources by *Bacillus megaterium* SRKP-3. Bioresour. Technol., 101: 705-711.
19. Gouda, M.K., A.E. Swellam and S.H. Omar, 2001. Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources. Microbiol. Res., 156: 201-207.
20. Reddy, S.V., M. Thirumala and S.K. Mahmood, 2009. Production of PHB and P (3HB-co-3HV) biopolymers by Bacillus megaterium strain OU303A isolated from municipal sewage sludge. World J. Microbiol. Biotechnol., 25: 391-397.
21. Thirumala, M., S.V. Reddy and S.K. Mahmood, 2010. Production and characterization of PHB from two novel strains of *Bacillus* spp. isolated from soil and activated sludge. J. Ind. Microbiol. Biotechnol., 37: 271-278.
22. Punrattanasin, W., 2001. Production of polyhydroxyalkanoates for biodegradable plastics using activated sludge biomass: system development. Ph.D. Thesis. Virginia Polytechnic Institute and State University, USA.
23. Chua, A.S.M., H. Takabatake, H. Satoh and T. Mino, 2003. Production of polyhydroxyalkanoates (PHA) by activated sludge treating municipal wastewater: effect of pH, sludge retention time (SRT) and acetate concentration in influent. Water Res., 37: 3602-3611.
24. Kasemsap, C. and C. Wantawin, 2007. Batch production of polyhydroxyalkanoate by low-polyphosphate-content activated sludge at varying pH. Bioresour. Technol., 98: 1020-1027.
25. Wen, Q., Z. Chen, T. Tian and W. Chen, 2010. Effects of phosphorus and nitrogen limitation on PHA production in activated sludge. J. Environ. Sci., 22(10): 1602-1607.
26. Rodgers, M. and G. Wu, 2010. Production of polyhydroxybutyrate by activated sludge performing enhanced biological phosphorus removal. Bioresour. Technol., 101: 1049-1053.
27. Lageveen, R.G., G.W. Huisman, H. Preusting, P. Ketelaar, G. Eggink and B. Witholt, 1988. Formation of Polyesters by *Pseudomonas oleovorans*: Effect of substrates on formation and composition of poly-I-3-hydroxyalkanoates and poly-I-3-hydroxyalkenoates. Appl. Environ. Microbiol., 54(12): 2924-2932.
28. Kimura, K., M. Yamaoka and Y. Kamisaka, 2004. Rapid estimation of lipids in oleaginous fungi and yeasts using Nile red fluorescence. J. Microbiol. Methods, 56: 331- 338.
29. Atlas, M., 1997. Handbook of Microbiological Media. 2nd ed., CRC Press, Boca Raton.
30. APHA, AWWA and WEF. 1998. Standard Methods for the Examination of Water and Wastewater. 19th ed., United Book Press, Baltimore.
31. Schlegel, H.G., R. Lafferty and I. Krauss, 1970. The isolation of mutants not accumulating poly-beta-hydroxybutyric acid. Arch. Microbiol., 71(3): 283-294.
32. Ostle, A.G. and J.G. Holt, 1982. Nile blue A as a fluorescent stain for poly-beta-hydroxybutyric acid. Appl. Environ. Microbiol., 44: 238-241.
33. Gorenflo, V., A. Steinbuchel, S. Marose, H. Rosenberg and T. Scheper, 1999. Quantification of bacterial polyhydroxyalkanoic acids by Nile red staining. Appl. Microbiol. Biotechnol., 51: 765-772.
34. Greenspan, P., E.P. Mayer and S.D. Fowler, 1985. Nile Red: a selective fluorescent stain for intracellular lipid droplets. J. Cell Biol., 100: 965-973.
35. Spiekermann, P., B.H.A. Rehm, R. Kalscheuer, D. Baumeister and A. Steinbuchel, 1999. A sensitive, viable-colony staining method using nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch. Microbiol., 171: 73-80.
36. Singh, M., S.K.S. Patel and V.C. Kalia, 2009. Bacillus subtilis as potential producer for polyhydroxyalkanoates. Microb. Cell Factories, 8: 38 doi:10.1186/1475-2859-8-38.
37. Aslim, B., F. Caliskan, Y. Beyatli and U. Gunduz, 1998. Poly-β-hydroxybutyrate production by lactic acid bacteria. FEMS Microbiol. Lett., 159: 293-297.
38. Van Loosdrecht, M.C.M., C.M. Hooijmans, D. Brdjanovic and J.J. Heijnen, 1997. Biological phosphate removal. Appl. Microbiol. Biotechnol., 48: 289-296.
39. Seviour, R.J., T. Mino and M. Onuki, 2003. The microbiology of biological phosphorus removal in activated sludge systems. FEMS Microbiol. Rev., 27: 99-127.
40. Tchobanoglous, G., F.L. Burton and H.D. Stensel, 2003. Wastewater Engineering: Treatment and Reuse. 4th ed., Metcalf and Eddy, Boston.
41. Sarioglu, M., 2005. Biological phosphorus removal in a sequencing batch reactor by using pure cultures. Process Biochem., 40(5): 1599-1603.

42. Kargi, F. and A. Uygur, 2004. Hydraulic residence time effects in biological nutrient removal using five-step sequencing batch reactor. Enzyme Microb. Technol., 35: 167-172.
43. Wong, C.H., G.W. Barton and J.P. Barford, 2003. The nitrogen cycle and its application in wastewater treatment. In: D. Mara and N.J. Horan (eds). The Handbook of Water and Wastewater Microbiology. Academic Press, London, pp: 427-440.
44. Takabatake, H., H. Satoh, T. Mino and T. Matsuo, 2002. PHA (polyhydroxyalkanoate) production potential of activated sludge treating wastewater. Water Sci. Technol., 45(12): 119-26.
45. Baei, M.S., G.D. Najafpour, H. Younesi, F. Tabandeh and H. Eisazadeh. 2009. Poly(3-hydroxybutyrate) synthesis by *Cupriavidus necator* DSMZ 545 utilizing various carbon sources. World Appl. Sci. J., 7(2): 157-161.
46. Dave, H., C. Ramakrishna and J.D. Desai, 1996. Production of Polyhydroxybutyrate by petrochemical activated sludge and *Bacillus* sp. IPCB-403. Indian J. Exp. Biol., 34: 216-219.
47. Thirumala, M., S.V. Reddy and S.K. Mahmood, 2009. Isolation and identification of some *Bacillus* spp. producing poly-3-hydroxybutyrate (PHB). J. Pure Appl. Microbiol., 3(2): 631-636.
48. Borah, B., P.S. Thakur and J.N. Nigam, 2002. The influence of nutritional and environmental conditions on the accumulation of poly-β-hydroxybutyrate in *Bacillus mycoides* RLJ B-017. J. Appl Microbiol., 92: 776-783.