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Accumulation of Intracellular Polyhydroxybutyrate in *Alcaligenes* sp. d_2 Under Phenol Stress

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Abstract *Alcaligenes* sp. d_2 isolated from soil was earlier reported as a potent phenol-degrading organism. In the Fourier transform/infrared spectroscopic analysis of the biodegraded sample, the aromatic stretching was missing and the spectrum gave evidence for the presence of polyhydroxybutyric acid along with its depolymerized products. In the gas chromatogram of the biodegraded sample, the peak of phenol at 14.997 min was absent and there were many peaks after 20 min. The organism could carry out 100% degradation of phenol in 32 h and could progressively result in early accumulation of polyhydroxybutyrate (PHB) intracellularly from 8 h onwards. The various conditions optimized for the maximum accumulation of intracellular PHB were pH 7.0, incubation time 24 h, phenol concentration 15 mg/100 ml, and ammonium sulfate concentration 25 mg/100 ml.

Keywords *Alcaligenes* sp. d_2 · Phenol · Biodegradation · Spectroscopic analysis · Polyhydroxybutyrate accumulation

Introduction

The large scale use of plastics and their improper disposal have created serious environmental problems. The need of the hour is to find alternative biodegradable materials with the physical and industrial properties similar to the petrochemically derived plastics [1]. Polyesters such as polyhydroxybutyrate (PHB) or other polyhydroxyalkanoates (PHAs) have attracted commercial and academic interest as new biodegradable materials. PHB is 100% biodegradable and can be processed like thermoplastics. It is nontoxic and water resistant. Polyhydroxybutyric acids are a group of storage compounds of carbon and energy that are accumulated during unbalanced growth by many bacteria. PHB is deposited intracellularly in the form of inclusion bodies and may account for up to 90% of the cellular dry weight [2, 3]. It is used in the manufacture of packaging films, bags, containers, biodegradable carriers for long-term storage of drugs, and ion-conducting polymers. It is

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used as a raw material for chiral compounds and also for blood vessel replacements [4]. Depending upon the utilized sources of carbon and nitrogen, PHB may be selectively induced in bacterial species [5].

In the present study, we discuss the PHB accumulation in the bacterium *Alcaligenes* sp. d_2 using phenol as the carbon source. This strain was earlier reported to be a potent phenol-degrading one [6].

Phenol and its derivatives are toxic and classified as hazardous materials [7]. Wastewaters originated from oil refineries, pulp and paper manufacturing plants, steel, and pharmaceutical industries contain phenols and phenolic compounds. As a water soluble compound, phenol is generally found to contaminate streams, rivers, and lakes that are situated near the industrial areas [8]. Wastewaters including phenols and phenolic compounds must be treated to prevent serious ecological risks. Biological treatment has been shown to be economical, practical, and most versatile approach as it leads to complete mineralization of phenol [9]. Many microorganisms are reported to use phenol as a sole source of carbon and energy including the species used in the present study [6]. PHB production could be considered as one of the solutions for the bioremediation of wastewaters containing phenolic compounds [10]. The objective of the study is to evaluate the production of PHB by *Alcaligenes* sp. d_2 using phenol as the sole source of carbon.

Materials and Methods

Microorganism The microorganism used in the present study was the bacteria *Alcaligenes* sp. d_2. It was isolated from a detergent contaminated soil and was reported as an efficient phenol-degrading organism [6].

Inoculum and Media Preparation One loopful of the culture was inoculated to 50 ml of peptone broth containing 7.5 mg phenol and incubated over night (30±2 °C, 150 rpm). The cells were harvested by centrifugation (10,000 rpm, 30 min, 4 °C). The pellets were collected and diluted using physiological saline (0.85% NaCl) till the optical density became 1. This was used as the inoculum for all studies. The medium used in the study was mineral salt phenol medium (MSPM) and the composition was as follows: KH_2PO_4—100 mg, $(NH_4)_2SO_4$—100 mg, $MgSO_4$ $7H_2O$—50 mg, and $CaCl_2$—1 mg in 100 ml of the medium. The pH was adjusted to 7.0.The solution was made up to 100 ml and was autoclaved at 121 °C for 20 min. Fifteen milligrams of phenol was added to the medium after sterilization.

Analysis of the Products of Biodegradation by FT/IR and GC/MS To the mineral salt phenol medium, taken as 1,000-ml aliquots, 3% inoculum was added and incubated on a shaker (30± 2 °C, 150 rpm). After 32 h of optimum incubation, the cells were removed by centrifugation at 10,000 rpm for 30 min. The supernatant was repeatedly extracted with solvent ether and concentrated by evaporation. This extract was used as the extract of biodegraded MSPM (biodegraded phenol). A concurrent control was kept without inoculation. It was also extracted, concentrated in the similar way, and was used as the extract of uninoculated MSPM (phenol control). The two extracts were subjected to Fourier transform/infrared spectroscopic (FT/IR) and gas chromatographic/mass spectroscopic analysis at Sophisticated Test and Instrumentation Centre, Cochin University, Cochin, Kerala, India.

In Vivo Detection of PHB by Fluorescent Microscopy The bacterial cells in MSPM medium were subjected to centrifugation at 10,000 rpm for 30 min at 4 °C. The supernatant was

discarded and the cell pellets were resuspended in 1 ml of sterile distilled water. Heat-fixed smear of the bacteria was stained with 1% aqueous solution of Nile blue A. This solution was heated for 10 min at 55 °C in a coplin-staining jar. The slide was washed with tap water and was placed in 8% aqueous acetic acid solution for 1 min. After washing, the slide was observed under an epifluorescence microscope using an exciter filter that provided an excitation wavelength of approximately 460 nm [11].

Recovery, Purification, and Assay of Intracellular Polyhydroxybutyrate Polyhydroxybutyrate was extracted, purified, and assayed as per the method suggested by Law and Slepecky [12]. PHB obtained from Sigma Aldrich was used as the standard.

Optimization of the Conditions for the Production of PHB

Incubation Period Of the mineral salt phenol medium (pH 7), 100-ml aliquots were inoculated with *Alcaligenes* sp. d_2 (3% inoculum) and incubated on a shaker (30±2 °C, 150 rpm). Cells were separated from these samples over a time period of 8, 16, 24, 32, 40, and 48 h, respectively, by centrifugation (10,000 rpm for 30 min) in a cold centrifuge and the pellet was assayed for PHB production as mentioned earlier.

pH Optimization of pH was done by conducting the PHB production at different pH of 3, 4, 5, 6, 7, 8, and 9 followed by the PHB assay.

Carbon Source Phenol was used as the sole source of carbon. PHB accumulation studies were conducted as mentioned above at various phenol concentrations of 3.75, 7.5, 11.25, 15, 18.75, 22.5, and 26.25 mg/100 ml. The highest concentration of phenol that gave maximum PHB accumulation was selected as the optimum condition.

Nitrogen Source Ammonium sulfate was used as a nitrogen source. PHB accumulation studies were conducted as mentioned above at various concentrations of 25, 50, 75, 100, 125, 150, and 175 mg/100 ml. After 24 h of incubation period, the samples were assayed for PHB accumulation.

Result and Discussion

Alcaligenes sp. d_2 utilized phenol as the sole source of carbon and it could achieve 100% phenol removal after 32 h at a pH range of 6–7 in mineral salt phenol medium [6]. On analyzing the similarity of the FT/IR spectrum of control (uninoculated MSPM) with National Institute of Standards and Technology library, the spectrum (Fig. 1) gave 83.33% match with that of phenol. The characteristic C = C ring stretch in aromatic compounds (1,593, 1,499, and 1,472 cm^{-1}), the aromatic C–H stretch (3,045 cm^{-1}), presence of O–H bond (1,361 cm^{-1}), and intermolecular H-bonded OH (3,286 cm^{-1}) clearly suggested the presence of phenol in the extract of the control. The FT/IR spectrum of the biodegraded phenol (Fig. 2) showed a shift of the band from 3,286 to 3,331 cm^{-1}. The band at 3,045 cm^{-1}, representing the aromatic stretching, was missing. The bands at 1,593, 1,477, and 1,472 cm^{-1} were also disappeared suggesting the absence of aromatic compounds in the biodegraded phenol. This indicated the ring cleavage and utilization of phenol by the organism.

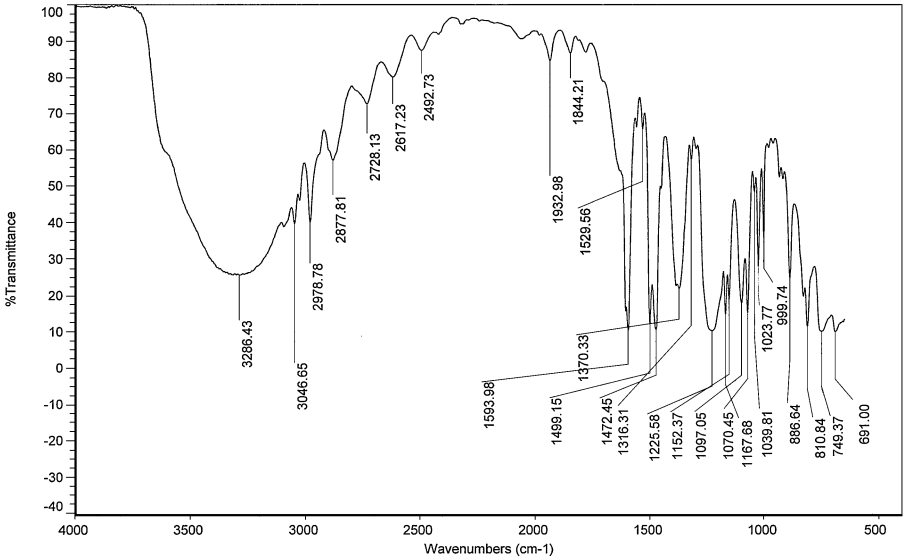

Fig. 1 FT-IR analysis of the extract of uninoculated MSPM (control)

Broad absorption bands at 3,333 cm^{-1} indicated the presence of polymeric substances in the extract. Library search of the FT/IR spectrum suggested 68.07% match with that of polyhydroxybutyrate. It followed that when *Alcaligenes* sp. d_2 was grown in mineral salt phenol medium, it could carry out both phenol degradation and PHB accumulation. Phenol was the only carbon source supplied. On 100% utilization of phenol within 32 h, the

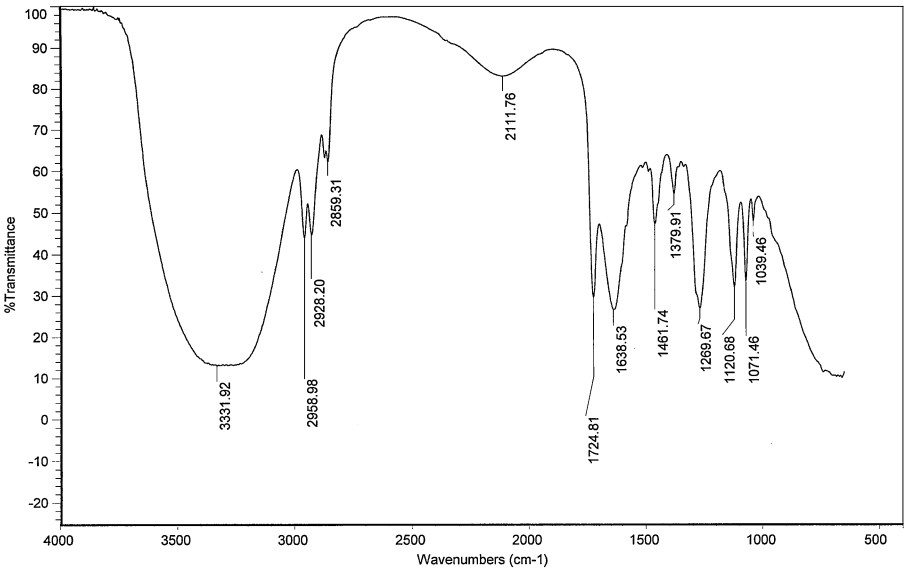

Fig. 2 FT-IR analysis of the extract of biodegraded MSPM

organism started utilizing its intracellularly stored energy source resulting in the release of depolymerized PHB [13].

The gas chromatogram of the control sample showed peaks at 2.951, 5.881, 7.271, 7.720, 14.997, 16.432, and 18.238 min. Mass spectroscopic analysis of the above peaks suggested that the peak at 14.997 min was that of phenol. Gas chromatogram of the biodegraded phenol extract showed peaks at 2.951, 5.88, 7.271, 7.720, and 16.432, 18.238, 23.394, 26.564, 36.204, 36.784, 37.962, 38.999, and 39.081 min (Fig. 3). The peak corresponding to phenol in the gas chromatogram of control (residence time 14.999) was totally absent in the chromatogram of biodegraded phenol. This confirmed that the phenol supplied in the medium was completely removed by *Alcaligenes* sp. d_2 under optimum conditions. The mass spectroscopic analysis of the additional peaks suggested the presence of high molecular weight fractions in the extract of the biodegraded phenol. The molecular weights of these fractions were very close. This again supported the hydrolysis of intracellular PHB upon complete utilization of phenol within 32 h resulting in the release of PHB hydrolyzed products.

Bacterial systems were known to synthesize PHB from acetyl coA [14]. PHB could be synthesized by condensation of two molecules of acetyl coA to acetoacetyl coA with a thiolase encoded by phaA, subsequent reduction to three hydroxybutyryl coA with a reductase encoded by phaB and polymerization to PHB with a synthase encoded by phaC [15]. The biodegradation of phenol resulted in the formation of acetyl coA and this might have induced the synthesis of PHB. In fact, the aliphatic polymeric substances like polyhydroxybutyric acids were usually accumulated and hydrolyzed intracellularly in many species of *Alcaligenes* under stress conditions [16].

Various conditions of nutritional limitation lead to the accumulation of PHB as an intracellular storage granule in bacteria. Later they may serve as both carbon and energy

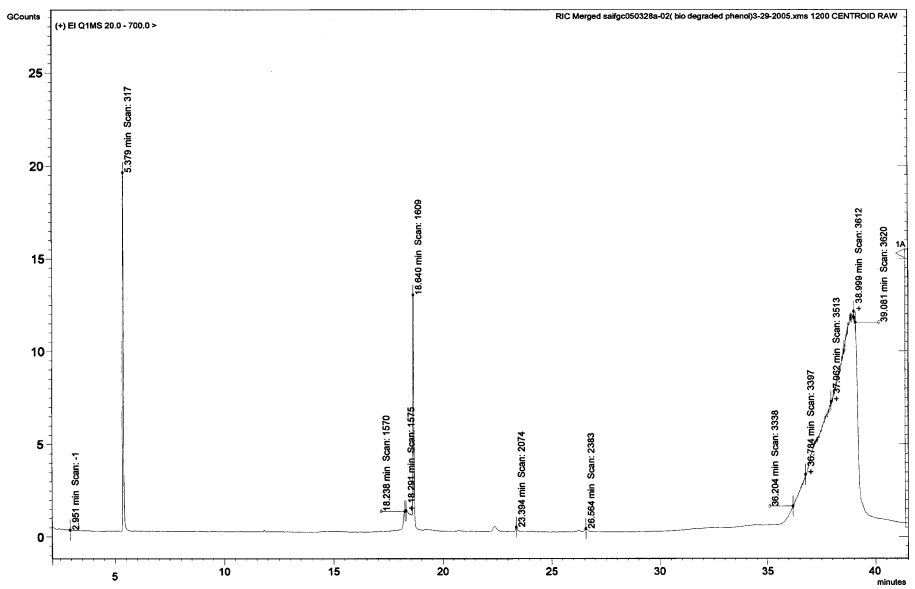

Fig. 3 Gas chromatographic analysis of the extract of biodegraded MSPM

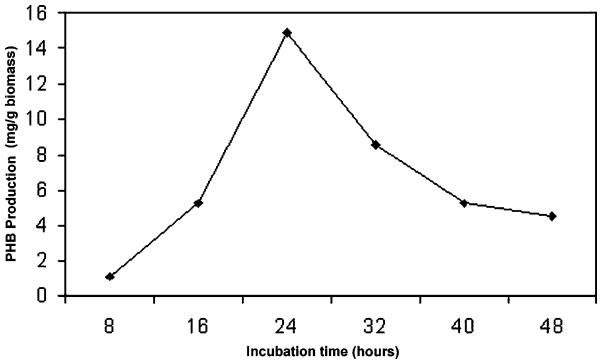

Fig. 4 Optimization of incubation period for intracellular polyhydroxybutyrate accumulation in *Alcaligenes* sp. d_2

source during starvation. The amount of PHB accumulated after 8 h of inoculation was 1.12 mg/g biomass (Fig. 4). In this study, the induction of PHB synthesis occurred at an earlier time than that of the previous report where the intracellular PHB synthesis started only after 12 h of incubation [17]. At 8 h of incubation time, the amount of phenol utilized by the organism was above 60%. The phenolic stress might have resulted in the early induction of intracellular PHB. The PHB accumulation was enhanced up to 3.5 mg/g biomass after 16 h of incubation. The maximum amount of PHB accumulation (14.93 mg/g biomass) took place at 24 h of incubation. However, there was progressive declining of the accumulated PHB from 32 h onwards. This was due to the complete utilization of the given carbon source, phenol. At 48 h of incubation, there was only 4.48 mg/g biomass of intracellular PHB.

The pH of the medium was found to be critical in the accumulation of intracellular PHB. The PHB accumulation was less at a pH range of 3–5 (Fig. 5). At a pH range of 6–7, there was increased PHB accumulation with a maximum of 20.29 mg/g biomass at pH 7. However, at alkaline pH, the PHB accumulation was very less.

On analyzing the effect of phenol concentration on PHB accumulation (Fig. 6), it was observed that a phenol concentration of 15 mg/100 ml was giving maximum intracellular accumulation of PHB (19.25 mg/g of biomass). This was also the optimum phenol concentration for biodegradation. Hence, the accumulation of the intracellular PHB was very much affected by the concentration of phenol in the medium. Lower concentration of phenol might be insufficient for the accumulation of PHB and higher concentration of phenol was found to be growth limiting [18].

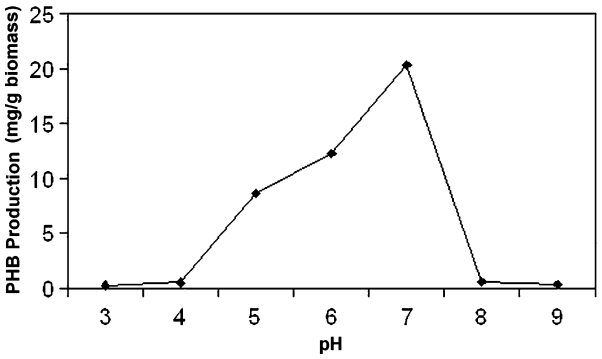

Fig. 5 Optimization of pH for the intracellular polyhydroxybutyrate accumulation in *Alcaligenes* sp. d_2

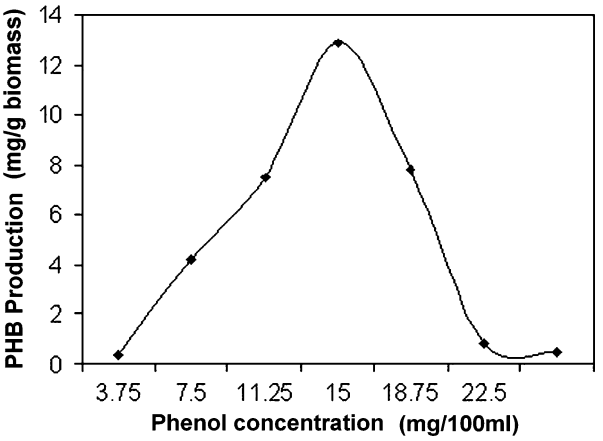

Fig. 6 Optimization of the phenol concentration in the media for the accumulation of intracellular polyhydroxybutyrate in *Alcaligenes* sp. d_2

Ammonium sulfate is considered as an ideal source of nitrogen for the intracellular accumulation of PHB [19]. In the mineral salt phenol medium, the ammonium sulfate concentration was kept at 100 mg/100 ml for phenol biodegradation. As nitrogen limitation had a profound effect on PHB accumulation [13], studies were conducted at lower concentrations of $(NH_4)_2SO_4$. PHB accumulation was found to be maximum (25 mg/g biomass) at lowest dosage of nitrogen source (25 mg/100 ml). On increasing the $(NH_4)_2SO_4$ concentration from 25 to 175 mg/100 ml at an interval of 25 mg, there was progressive and considerable reduction in the intracellular accumulation of PHB (Fig. 7). During nitrogen limitation, NADH/NAD values increase leading to increased PHB production. PHB accumulation could be enhanced by facilitating the metabolic flux of acetyl coA to PHB synthetic pathway [20].

Alcaligenes sp. d_2 used in this study is a highly promising strain for the production of PHB. Studies are going on to bring about the mechanism of PHB accumulation and utilization. This strain was successfully used for the laboratory scale treatment of phenolic wastewaters [21]. Further work is in progress to scale up the production of PHB using phenolic effluents. Once appropriate technology is developed, the strain can be industrially exploited for both phenolic wastewater treatment and large scale production of PHB.

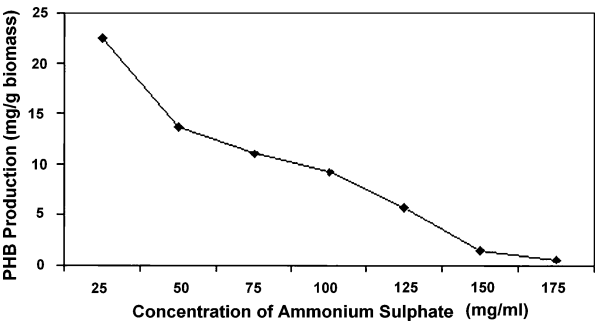

Fig. 7 Optimization of ammonium sulfate concentration for the accumulation of intracellular polyhydroxybutyrate in *Alcaligenes* sp. d_2

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References

1. Sujatha, K., Mahalakshmi, A., & Shenbagarathai, R. (2005). *Indian Journal of Biotechnology*, *4*, 323–335.
2. Ohura, T., & Kasuya, K. D. Y. (1999). *Applied and Environmental Microbiology*, *15*, 189–197.
3. Uchino, K., Saito, T., & Jendrossek, D. (2008). *Applied and Environmental Microbiology*, *74*(4), 1058–1063.
4. Madison, L. L., & Huisman, G. W. (1999). *Microbiology and Molecular Biology Review*, *63*, 21–53.
5. Sudesh, K., Abe, H., & Doi, Y. (2000). *Progress in polymer science*, *25*, 1503–1555.
6. Nair, C. I., & Shashidhar, S. (2004). *Soil Science*, *3*(4), 47–51.
7. Zumrye, A., & Gultac, B. (1999). *Enzyme and Microbial Technology*, *25*, 344–348.
8. Prpich, G., & Daugulis, A. (2005). *Biodegradation*, *16*(4), 329–339.
9. Nuhoghu, A., & akin, B. (2005). *Process Biochemistry*, *40*, 233–239.
10. Ramos-Cormenzana, A., Monteoliva-Sanchez, M., & Lopenz, M. S. (1995). *International Journal of Biodeterioration and Biodegradation*, *35*, 249–268.
11. Ostle, A. G., & Holt, J. G. (1982). *Applied and Environmental Microbiology*, *44*, 238–241.
12. Law, J. H., & Slepecky, R. A. (1961). Assay of poly-β-hydroxybutyric acid. *Journal of Bacteriology*, *82*, 33–36.
13. Thakor, N. S., Patel, M. A., Trivedi, U. B., & Patel, K. C. (2003). *World Jounal of Microbiology and Biotechnology*, *19*, 185–189.
14. Thakor, N., Trivedi, U., & Patel, K. C. (2006). *Indian Journal of Biotechnology*, *5*, 137–147.
15. Uchino, K., Saito, T., Gebauer, B., & Jendrossek, D. (2007). *Journal of Bacteriology*, *189*(22), 8250–8256.
16. Anderson, A. J., & Dawes, E. A. (1990). *Microbiological Reviews*, *54*, 450–472.
17. Saegusa, H., Shiraki, M., Kanal, C., & Saito, T. (2001). *Journal of Bacteriology*, *183*(1), 94–100.
18. Hao, O. J., Kim, M. H., Seagren, E. A., & Kim, H. (2002). *Chemosphere*, *46*, 797–807.
19. Yuksekdag, Z., & Abeyatti, Y. (2008). *Journal of applied Biological Sciences*, *2*(2), 7–10.
20. Lee, Y., Kim, M. K., Chang, H. N., & Park, Y. H. (2006). *FEMS Microbiology letters*, *131*(1), 35–39.
21. Nair, C. I., Jayachandran, K., & Shashidhar, S. (2007). *Bioresource Technology*, *98*, 714–716.