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RESPONSE SURFACE METHODOLOGY OPTIMIZATION OF POLYHYDROXYALKANOATE BY RECOMBINANT *Bacillus megaterium* pPSPHAR1/1 STRAIN USING FISH PROCESSING WASTE PRODUCTION

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Abstract. Polyhydroxyalkanoates (PHAs) are biomaterials that are accumulated intracellularly by bacterial cells in response to nutrient imbalances under environmental stress. Polyhydroxybutyrate (PHB) is a bioplastic that is of interest to research to find an alternative to fossil-derived plastics. The optimal physical and nutritional conditions for PHB production were investigated by varying one variable at a time. To achieve maximum PHA production, the culture conditions for *B. megaterium* pPSPHAR1/1 were optimized through response surface methodology (RSM). The final optimum fermentation conditions included: 13.34 (g/L) glucose; 7.28 (g/L) Na_2HPO_4; 4.45 (g/L) K_2HPO_4; $MgSO_4$ 0.2; 2 (g/L) $(NH_4)_2SO_4$; NH_4Fe(III) citrate 0.005 %; acid citric 0.1 %; 2 Ml of trace minerals, 3 (%w/v) fish oil; 1.3 (%v/v) fish extract; inoculum size, 10 % (v/v)and temperature of 37 ^oC for 72 h. Using the optimal medium, the PHB production of this recombinant strain accumulated a PHB content of about 76.2 % per cell dry weight in a 5 L stirred bioreactor.

Keywords: Bacillus megaterium, Polyhydroxybutyrate, PHB, submerged fermentation, fish processing waste, oil fish.

Classification numbers: 2.3.1, 1.1.5, 3.7.2**,** 3.3.2

1. INTRODUCTION

For a long time, the problem of plastic waste has become a threat to the ecological environment around the world, it is estimated that every year millions of tons of plastic cannot be processed and cause serious pollution to the living environment [1]. Most of the waste plastic products are difficult to biodegrade and they accumulate in the ecosystem, resulting in a significant burden on solid waste management. To reduce the demand for plastic products made from petroleum-based plastics, bio-based plastics or degradable polymers will be used in the future.

Polyhydroxybutyrate (PHB) is one of the short-chain PHAs and has been the best-studied PHA. PHB was the first PHA with commercial potential as a biodegradable thermoplastic and a biomaterial [2]. PHB is used as a carbon and energy reserve produced by microorganisms and its synthesis is favored by environmental stresses such as nitrogen, phosphate or oxygen limitations [3]. PHB and other PHAs are synthesized and deposited intracellularly in granules and can amount to 30 - 90 % of the cellular dry weight [4]. Accumulation of intracellular storage PHAs considered a strategy of bacteria allowing their survival in different environments.

Polyhydroxybutyrate (PHB) is among the most well-known, recognized as completely biosynthetic, biodegradable and biocompatible. It can be used in medicine and is produced from various renewable resources [2, 5]. PHBs are energy particles that are accumulated intracellularly by microorganisms to adapt to harsh environmental conditions, so PHB is also easily degraded by microorganisms to form water and CO_2 [6]. PHB which is mainly produced from the genus *Bacillus* can accumulate up to 30 - 50 % of the cell dry weight [5, 7].

Vietnam's export of pangasius and basa fish is increasing every year. So the source of fish processing by-products is quite large, accounting for 55 - 64 % of processed fish output, including head, bones, skin, intestines, liver, blood, and fins. Fish waste is proved to be a great source of minerals, containing 58 % protein and 19 % fat. Normally, it will be classified used as supplementary feed for livestock and poultry; a large part of fish fat and fish skin is recovered for processing to produce fish oil and industrial collagen. It is also a source of nutrient-rich substrates suitable for the growth of bacreria as well as the development and industrial-scale production of PHB [8]. Comparably, the world's use of petroleum-based plastics and fish processing waste cannot decrease, only increases every year and will increase the pollution burden on the environment. Therefore, applying microbial fermentation to fish processing waste to produce biopolymers on an industrial scale is attracting growing interest as new raw material and a low-cost process [9].

In this study, we used the optimization method of fermentation medium from fish oil and fish extract for the recombinant bacterial strain *B. megaterium* pPSPHAR1/1 to biosynthesize PHB. This work can reduce the cost of PHB production, instead of expensive pure chemicals, we used domestic low cost and renewable resources including fish production waste (fish oil and fish extract) as carbon and nitrogen sources to produce PHB by *B. megaterium* pPSPHAR1/1 strain. And therefore we can reduce the cost of producing PHB on an industrial scale, promote the use and production of bioplastics that completely replace petroleum-derived plastics, thereby reducing environmental pollution.

2. MATERIALS AND METHODS

2.1. Bacterial strains and media

Microorganism: The recombinant *Bacillus megaterium* pPSPHAR1/1 strain was obtained from the collection of microorganisms of the Department of Animal Cell Biotechnology, Institute of Biotechnology, Vietnam Academy of Science and Technology.

Culture media: An LB agar medium was created with 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L agar at pH 7. A modified mineral medium for accumulation PHA production of pPSPHAR1/1was used for the fermentation: 14.32 g/L glucose; 7.28 g/L Na_2HPO_4; 4.45 g/L K_2HPO_4; 0.2 g/L $MgSO_4$; 2 g/L $(NH_4)_2SO_4$; 0.005 % NH_4Fe(III) citrate ; 0.1 % acid citric; 2 ml/L trace minerals; at pH 7 [8]; The trace mineral included 10 mg/L $ZnSO_4.7H_2O$; 3 mg/L $MnCl_2.4H_2O$, 30 mg/L H_3PO_4, 20 mg/L $CoCl_2.6H_2O$, 1 mg/L $CuCl_2.2H_2O$,

2 mg/L $NiCl_2.6H_2O$, and 3 mg/L $Na_2MoO_4.2H_2O$. Sugars and mineral salt solutions were autoclaved separately at 121 °C for 20 min.

Additional substrates

*Table 1.*Additional substrates for screening bacteria in this study.

Substrates	Dilute solutions	Stock concentrations	Final concentration
Tetracylin (Sigma-Aldrich)	H_2O deion	50mg/mL	10 µg/mL
D-Xylose (Sigma-Aldrich)	H_2O deion	500mg/mL	15mg/mL

2.2. Methods

2.2.1. Preparation of fish solid waste extract

The fish solid waste (FSW) extracts were used as the substrate for *B. megaterium* pPSPHAR1/1 to reduce the cost of PHA production. The FSW including scales, intestine, etc. of *Pangasianodon hypophthalmus* was collected from Hasa seafood corporation, Can Tho, Viet Nam. It was washed three times by distilled water and then stored at –20 °C until use. The FSW was mixed with distilled water at a ratio of 1:1 (w/v) and boiled at 50 °C for 120 min. Then supernatants (fish oil and fish extract liquid were filled to remove insoluble materials and cell debris [8]. The extracted samples were stored at –20 °C. The mineral medium was supplemented with FSW extract in 1 % (v/v) fish extracted solution and 2% (w/v) fish oil to formulate PHB production.

2.2.2. Optimization of medium componentsfor PHA production

*Table 2.*Test variables and levels of CCD for the optimization of glucose, fish oil, and fish extract for the polyhydroxybutyrate production by *Bacillus megaterium* pPSPHAR1/1 in triangle flasks.

Factors	Symbols	Unit	Low	High Actual	Low	Middle	High
Glucose	A	***g/l***	10	15	– 1	0	1
Fish extract	B	***% v/v***	0.1	2	– 1	0	1
Fish oil	C	***%, w/v***	0.5	3	– 1	0	1

The recombinant R1/1 strain was preliminarily investigated for the influence of some factors such as glucose, fish fat, fish extract and the experimental thresholds of these 3 factors were selected appropriately to conduct optimization by response surface method, giving the results as presented in Table 2. The PHA production from the recombinant strain R1/1 was optimized by Response Surface Methodology based central composite design (CCD) (Design Expert 7.1.5, Stat-Ease Inc., Minneapolis, MN). Three factors were used to design the experimental combination represented as at high (+1) and low (-1) levels. The design was used to find the optimum carbon (glucose, fish oil) and nitrogen (fish extract) sources. The flasks were operated according to the factors combined by the small factorial CCD (Tables 2 and 3).

Experiments were conducted in a 500 mL triangle flask (containing 150 mL of medium). Each experiment was repeated 3 times.

2.2.3. Extraction and quantitative analysis of PHA

The culture medium was inoculated and maintained at 37 ^{o}C and 150 rpm for 72 h. PHA extraction and quantitative analysis were performed using a previously described method [8]. The PHA concentration was determined by measuring the absorbance at 235 nm by crotonic acid method [11]. The results were compared with the standard curve plotted between concentrations of crotonic acid by PHB (Sigma-Aldrich)under the same conditions. The percentage of PHA accumulation of *B.megaterium* pPSPHAR1/1 was estimated as the percentage composition of PHA present in dry cell weight (DCW), which was calculated using the following formula:

$$\text{PHA}(\%) = \frac{\text{amount of dry extracted PHA (g/L)}}{\text{DCW (g/L)}} \times 100.$$

2.2.4. Structural characterization of PHB

NMR Analysis: The molecular mobility of PHA was confirmed by proton nuclear magnetic resonance (1H-NMR) spectroscopy. The 1H-NMR spectra of the PHA sample were recorded in $CDCl_3$ on a Bruker ACF 300 spectrophotometer at 300 MHz using "Tetramethylsilane" as the internal standard [8, 10].

FT-IR Analysis: To identify the functional group that represents signal peaks of the extracted PHA was subjected to FTIR analysis**.** In this experiment, 2 mg of PHA sample was mixed thoroughly with KBr powder (spectral grade) to make a KBr pellet. The pellet was dried at 100 °C for 4 h. The presence of functional groups of the PHA sample was recorded using a single beam spectrometer between wave numbers of 400 and 4000 cm^{-1} using Perkin Elmer spectrophotometer [8, 12].

2.2.5. Data processing

The experimental designs and regression analysis of the experimental data were examined and collected from Design-Expert software version 7.1.5 (Stat-Ease Inc., Minneapolis, USA). The surface quadratic model was checked by the analysis of variance (ANOVA). The quality of the polynomial model equation was judged by determining the coefficient R^2 and then analyzed by the F-test. Statistical analysis of the average value of the experimental data was carried out by Microsoft excel 2007. The whole experiment was repeated three times.

3. RESULTS AND DISCUSSION

3.1. Optimization of culture media based on central composite design (CCD)

There are many culture factors affecting the ability to accumulate PHA of the recombinant strain R1/1 such as glucose,K_2HPO_4, KH_2PO_4, yeast extract, fish extract, andoil fish fish oil?, etc. However, in this study, we only presented the results of the optimization of three factors: glucose, fish oil and fish extract based on CCD. The remaining ingredients of the medium were used according to the optimally evaluated contents (resultsare not shown here). Experiments were conducted in a 500 mL flask containing 150 mL of culture media with different

concentrations of glucose, fish oil and fish extract. The maximum content of PHA produced by *B. megaterium* pPSPHAR1/1was then analyzed. Table 3 lists the results for the yielded PHA contents ranging from 135 to 753 (mg/g CDW) from different 20 experiments.

Table 3. The production of PHAby *B. Megaterium*pPSPHAR1/1is affected by three factors based on CCD.

Std	Run no.	Factor			PHA, mg/g CDW
		A: Glucose, g/L	B: Fish extract, % v/v	C: Fish oil, %w/v	
15	1	12.5	1.05	1.75	753
18	2	12.5	1.05	1.75	711
14	3	12.5	1.05	3.9	731
6	4	15	0.1	3	632
17	5	12.5	1.05	1.75	689
5	6	10	0.1	3	291
20	7	12.5	1.05	1.75	751
11	8	12.5	0.0	1.75	423
3	9	10	2	0.5	281
16	10	12.5	1.05	1.75	744
8	11	15	2	3	601
7	12	10	2	3	548
2	13	15	0.1	0.5	410
4	14	15	2	0.5	562
1	15	10	0.1	0.5	213
19	16	12.5	1.05	1.75	618
12	17	12.5	2.6	1.75	489
9	18	8.3	1.05	1.75	135
10	19	16.7	1.05	1.75	458
13	20	12.5	1.05	0.5	457

3.2. Analysis of variance (ANOVA) for the quadratic model of PHA production from*Bacillus megaterium* pPSPHAR1/1

Table 3 presents the results in the form of analysis of variance (ANOVA) and the measurement of the F value and p-value. The p-value helps to understand the pattern of mutual interaction between the best variables. The smaller the p-value (p-value < 0.05), the larger the significance of the corresponding coefficient. In this case, the p-value of the model is equal to 0.0003 and the F value of the model is 11.6. These results show that the model is significant. The effect of glucose (A), fish extract (B) and fish oil (C) on the PHA production arehighly significant ($p = 0.0006$ for glucose factor, $p = 0.0223$ for fish waste source, and $p = 0.0042$ for fish oil source). Sothe effect of A^2 and B^2 on the PHB accumulation is also significant with a

probability value of $p < 0.05$. However, the C^2 (the effect of C^2?) is not significant ($p = 0.2281$). The interaction among these three sources (glucose and fish extract, glucose and oil extract, and fish extract and fish oil) shows a significant effect (Table 4). The lack of a fit F-value of 3.4 implied that it was non-significant relative to the pure error. There is a 10.42 % chance that such a large "Lack of Fit F-value" could occur due to noise. Responses such as PHA yield were studied and the overall second-order polynomial equations for PHA production are given below:

$$Y = 578.1*A + 436.9*B + 185.5*C - 20.8*A^2 - 113.1*B^2 - 21.2*C^2 - 3670.53.$$

Table 4. Analysis of variance (ANOVA) results for the effect of three factors (glucose, fish waste, and fish oil) on PHA production.

Source	Sum ofSquares	df	MeanSquare	FValue	p-value, Prob > F
Model	621114.4	9	69012.7	11.6	0.0003
A-Glucose	146654.9	1	146654.9	24.7	0.0006
B-Fish waste	43310.5	1	43310.5	7.3	0.0223
C-Fish oil	80893.7	1	80893.7	13.6	0.0042
AB	5202.0	1	5202	0.9	0.3713
AC	882.0	1	882	0.1	0.7080
BC	4.5	1	4.5	0.0	0.9786
A^2	246949.1	1	246949.1	41.6	< 0.0001
B^2	95233.2	1	95233.2	16.0	0.0025
C^2	9787.3	1	9787.3	1.6	0.2281
Residual	59380.2	10	5938		
Lack of Fit	45794.2	5	9158.8	3.4	0.1042
Pure Error	13586.0	5	2717.2		
Cor Total	680494.6	19			

Incomparison to the culture factors, the glucose factor shows the most affecting on the accumulation of PHA production. The lowest PHA value of 135 mg/g CDW was obtained from run No. 18 with the lowest glucose content (Table 3). In this experiment, the highest PHA content was 767.584 mg/g CDW when the flag was added to the contour model with the highest glucose content. The optimum medium for PHA production in *B. megaterium* pPSPHAR1/1cultivation was 13.34 g/L glucose, 3 % w/v fish oil, 1.30 % v/v fish extract, 7.28 g/L Na_2HPO_4, and 4.5 g/L K_2HPO_4 (Fig. 1). The highest PHA production of *B. subtilis* G-3 using rice bran as a substrate was 0.81 g/L [9]. However, the yield of PHAs of *B. megaterium* VB89 using similar culture media of *B. megaterium* pPSPHAR1/1 was 0.672 g/L [13].

To see the interaction of different coefficients for PHA accumulation by pSPHAR1/1, the graphs were plotted by Design Expert 7.1.5. A combined effect of the glucose, fish extract and fish oil had a positive influence on the PHA yield (Fig. 2).

Using ANOVA, the suitability of the model was confirmed by a satisfactory R^2 value of 0.9127, which means that 91.27 % of the variability in the response could be explained by the model and that 9 % of the variations occur while performing the experiments, thus indicating a

realistic fit of the model to the experimental data influencing the PHA production (Fig. 3). This assumption was confirmed by the observed vs the predicted results for the PHA production.

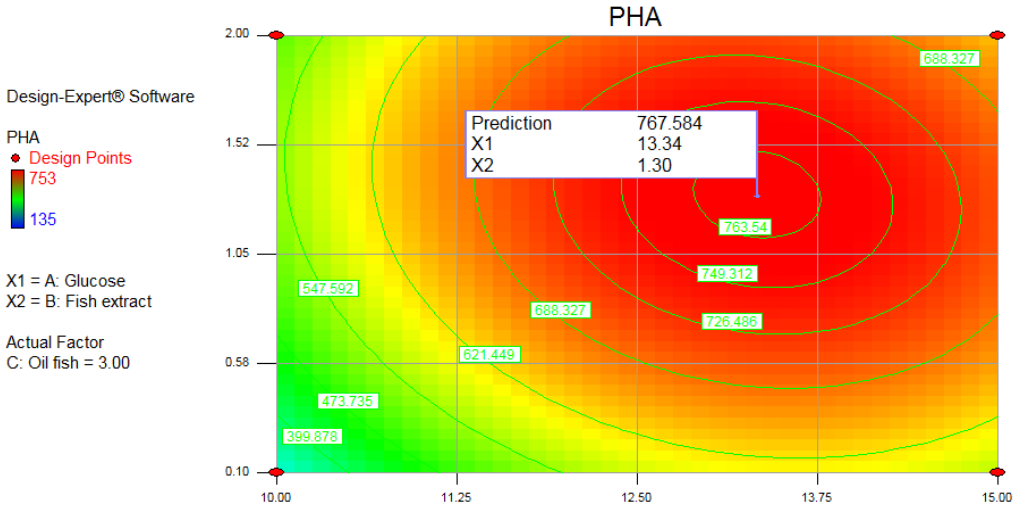

*Figure 1.*Three-dimensional (3D) contour plots of the maximal PHA production.

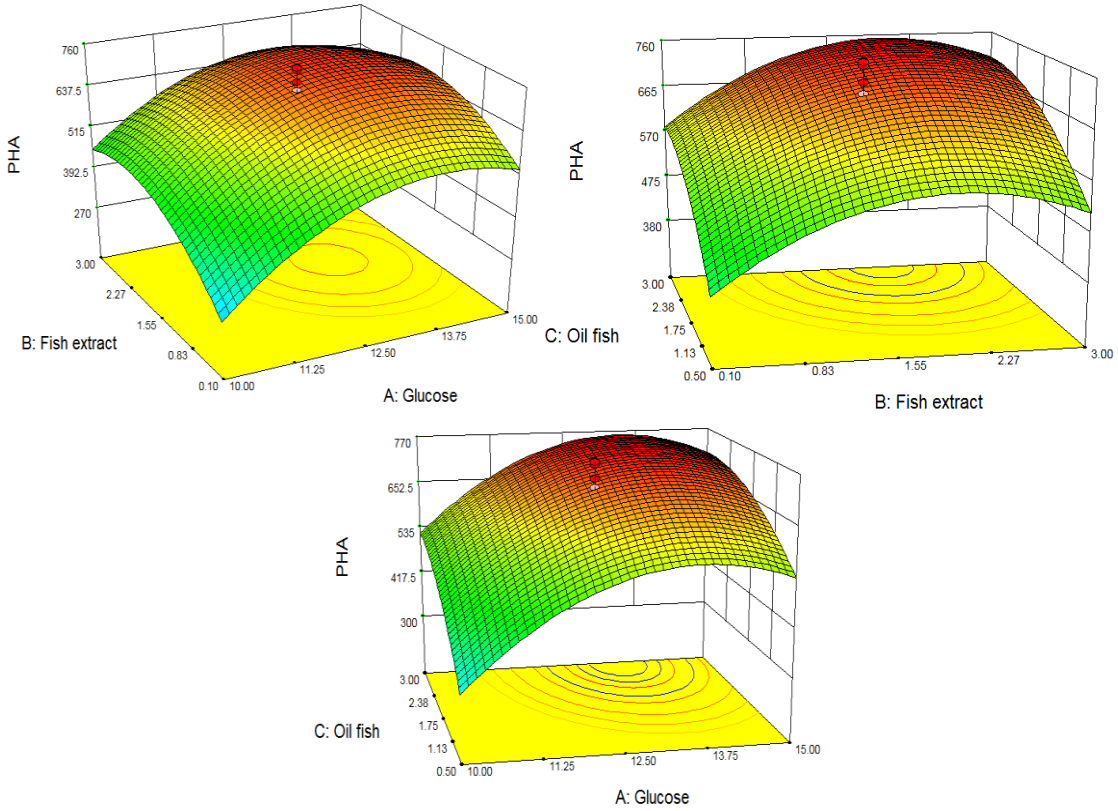

Figure 2. Three-dimensional (3D) response surface generated by the model for two variables that affect the yield of PHA; fish extract and glucose (a) and fish oil and fish extract (b) and fishoil and glucose.

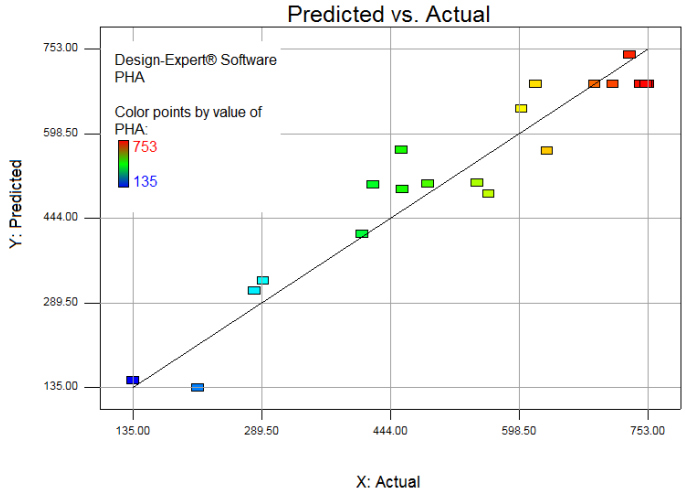

Figure 3. The graph showing the actual *vs* the predicted values under optimized conditions of *Bacillus megaterium* pPSPHAR1/1 for PHA accumulated activity.

3.3. The effect of time on the PHA production of *B. megaterium* pPSPHAR1/1 in optimized medium

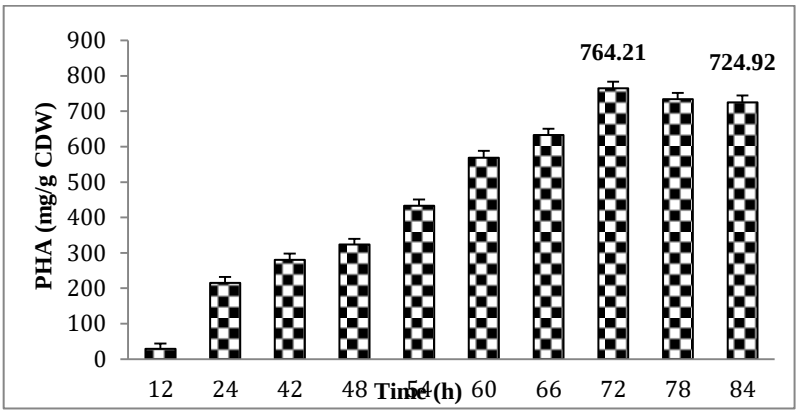

*Figure 4.*Time dependence of PHA production of *B. megaterium* pPSPHAR1/1 in optimized medium. Data shown are the mean of duplicate tests.

Experiments were carried out in a five liter stirring bioreactor using the determined optimum concentrations of 13.34 g/L of glucose, 3 % (w/v) fish oil, 1.3 % v/v fish extract, 4.45 g/L K_2HPO_4, 7.28 g/L Na_2HPO_4, 0.2 g/L $MgSO_4$; 2 g/L $(NH_4)_2SO_4$; 0.005 % NH_4Fe(III) citrate; 0.1 % acid citric; 2 mL of mineral trace, at pH 7. In these experiments, a part of the carbon source (glucose) was replaced with fish oil, and the fish extract was replaced with allof the yeast extract as a nitrogen source. This significantly reduces the cost of PHB production when using fish waste for submerged fermentation by *B. megaterium* on an industrial scale. Before optimization, the mineral medium containing 10 g/L glucose, 2 % (w/v) fish oiland 1 % fish extract in a flask was shaken at 150 rpm for 72 hours, and the PHA content was 565.4 ± 2.27 mg/g CDW. Figure 4 shows the growth pattern of *B. megaterium* pPSPHAR1/1 in the predicted optimal medium in a 5 L bioreactor. A maximum of 764.21 mg/g CDW PHA was obtained

using optimal concentrations after 72 hours. However, it should be affirmed that, although the amounts of PHA were less than in other studies, the predictions were the highest values achieved throughout the study. The PHA production of this study was in agreement with Biglari *et al*. (2020) who reported that the highest PHA concentration and CDW were achieved after 66 hours of cultivation [14].

Mohanrasu *et al*. (2020) has recently reported that culturing with glucose as a carbon source of *B.megaterium* could accumulate the maximal PHA production of 2.74 g/L after 72 hour of cultivation [15]. The actual amount of PHB production in the experiment of *B. drentensis* strain BP17 after 72 hours of cultivation reached 3.9 g/L on cell dry weight (CDW) [16].

3.4. Characterization of the purified PHA produced from *B. megaterium* pPSPHAR1/1

The PHA extracted from *B. megaterium* pPSPHAR1/1 was purified and analyzed by FTIR and NMR for chemical structure properties.

3.4.1. FTIR analysis

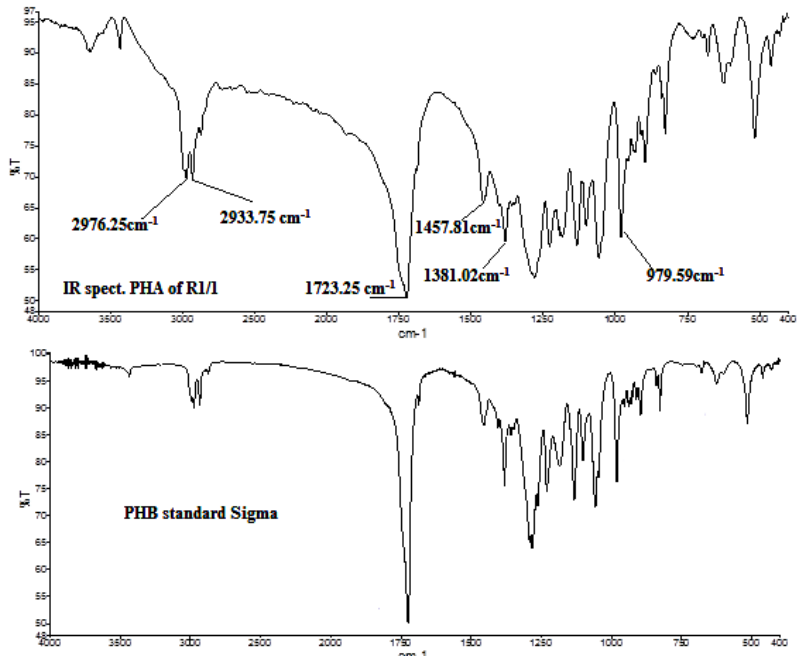

Figure 5. FTIR spectrum of PHA produced by*B. megaterium* pPSPHAR1/1 and of standard PHB (Sigma).

The functional group of the purified PHA from *B. megaterium* pPSPHAR1/1 was identified as C=O group by FTIR spectroscopy. IR analysis could help to better understand the chemical structure of PHA polymers and monomeric units. The IR spectrum showed two intense absorption bands at 1723 and 1506 cm^{-1} corresponding to the ester carbonyl group (C=O) and C-O stretching group, respectively [17]. These peaks are the biggest peaks in the spectra compared to those of the commercial PHB (sigma) (Fig. 5). The bands between 2976 and 2933 cm^{-1} correspond to the C-H stretching bonds of methyl (CH_3) and methylene (CH_2) groups [18]. The absorption bands at 1457 and 1381 cm^{-1} are attributed to the methyl group. A peak at 979

cm^{-1} corresponds to the presence of alkyl halides in the extracted polymer [18]. This result suggested the presence of polyhydroxybutyrate (PHB) as a common homopolymer of PHAs.

3.4.2. NMR analysis

The ^{1}H NMR spectra obtained from purified PHA produced by *B. megaterium* pPSPHAR1/1 under optimized cultivation is compared with the commercial PHB (Sigma, Aldrich Chemical, USA) (Fig. 6).

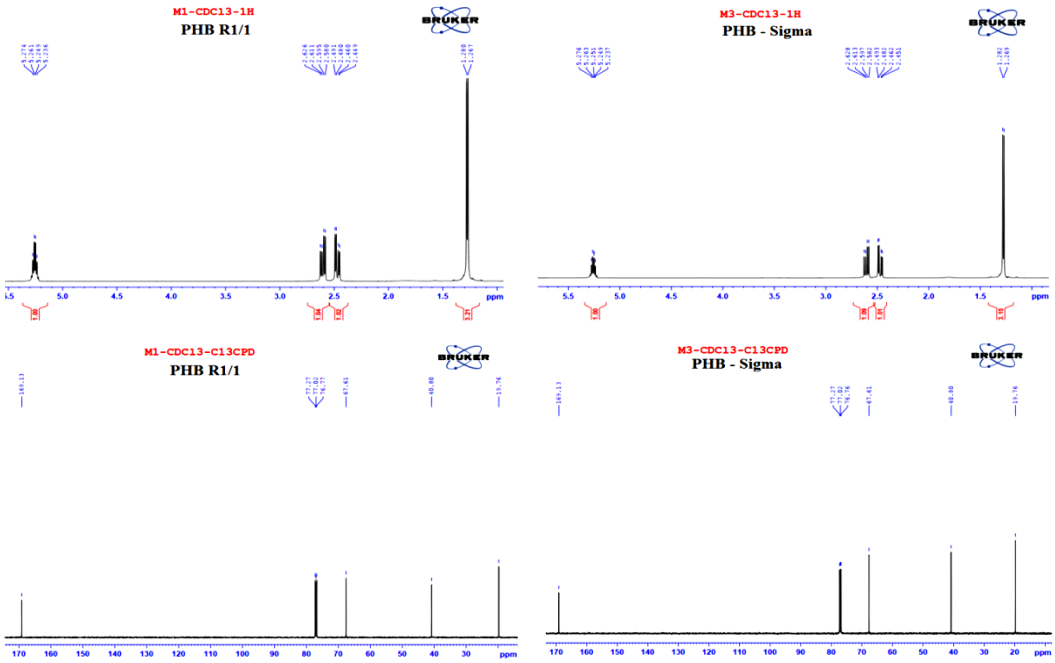

Figure 6. ^{1}H (above) and ^{13}C (below) NMR spectra of the purified PHA of *B.megaterium* pPSPHAR1/1 and standard PHB from Sigma.

Both spectrums were in perfect agreement with each other. The peak from 1.27 to 1.28 ppm corresponds to the terminal methyl group (CH3) (3H, d, J = 6.5 Hz).The spectra ranging from 2.45 to 2.62 ppm (1H, d, J = 5.5, 15.5 Hz and 1H, d, J = 7.5, 15.5 Hz) indicatethe methylene group (CH_2). The methine group (CH) of PHB is present from 5.24 to 5.27 ppm (1H, six, J = 6.5 Hz). The NMR spectrum of PHA from the strain pPSPHAR1/1 showed patterns similar to those of the published PHB [19]. Jan *et al*. (1996) reported that the peak at 1.0 ppm and 4.75 ppm showed the specific peak of water [20]. The ^{13}C NMR spectrum measured in $CdCl_3$ at 125 MHz confirmed the structure of PHB from *B. megaterium* pPSPHAR1/1. There are four peaks that are the signal of 4 carbon groups including 1 methyl group (δC 19.76), 1 methylene group (δC 40.80), 1 oxygen-linked methine group (δC 67.61), and 1 ester carbonyl group (δC 169.13). These findings confirm that the PHAaccumulated by *B. megaterium* pPSPHAR1/1 in the present work is indeed PHB.

4. CONCLUSION

Preliminary investigations revealed that the highest biomass and PHB concentrations could be achieved by using fish processing waste. Therefore, to improve bacterial growth and PHB production, fish production waste was used to boil before being added to the culture media. In

order to reduce the cost of PHB production, instead of using expensive pure chemicals, in this work, domestic low cost and renewable resources including fish processing waste were used to produce PHB by *B. megeterium* pPSPHAR1/1. Subsequently, RSM was used to optimize the medium composition with 3 % w/v fish oil; 1.3 % v/v fish extract and 13.3 g/L glucose to enhance PHB production of this recombinant strain. Using the optimal medium in a 5 L stirred-tank bioreactor, PHB production of this recombinant strain was increased to 764.2 mg/CDW. However, when compared to the previous experiments, the results obtained in the optimal medium indicated that the new composition was able to stimulate PHB synthesis in the optimal medium. Therefore, although the results of the present study may inspire industrial-scale biotransformation of renewable low cost resources, more investigations are needed to assess how PHB synthesis can be stimulated in *B. megaterium* pPSPHAR1/1 when growing in the medium composed of fish oil and fish extract as a part of the carbon and nitrogen sources.

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CRediT authorship contribution statement. Author 1: Formal analysis, writing original draft preparation. Author 2: Methodology, writing review and editing. Author 3: Formal analysis. Author 4: Supervision, Conceptualization. Author 5: Validation, Writing review and editing.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

1. Chanprateep S. - Current trends in biodegradable polyhydroxyalkanoates, Journal of Bioscience and Bioengineering **110** (6) (2010) 621-632.
2. Bernard M. - Industrial potential of polyhydroxyalkanoatebioplastic: a brief review, University of Saskatchewan Undergraduate Research Journal **1** (2014) 1-14.
3. Lee I. Y., Chang H. N.,and Park Y. H. - A simple method for recovery of microbial poly β-hydroxybutrate by alkaline solution treatment, Journal of Microbiology and Biotechnology **5** (1995) 238-240.
4. Gouda M. K., Swellam A. E., andOmar S. H. - Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources, Microbiological Research **156** (2001) (3) 201-207.
5. Kumar T., Singh M., Purohit H. J., *et al*. - Potential of Bacillus sp. to produce polyhydroxybutyrate from biowaste,Journal of Applied Microbiology **106** (6) 2017-2023.
6. Suriyamongkol P., Weselake R., Naraine S., Moloney M., Shah S. - Biotechnological approaches for the production of polyhydroxyalkanoates in microorganisms and plantsa review, Biotechnol Adv. **25** (2007) 148-175.
7. Venkateswar-Reddy M., Mawatari Y., Yajima Y., Satoh K., Venkata-Mohan S., Chang Y. C. - Production of poly-3-hydroxybutyrate (P3HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) P(3HB-co-3HV) from synthetic wastewater using Hydrogenophag-apalleronii, Bioresour. Technol. **215** (2016) 155-162.
8. Mohapatra S., Sarkar B., Samantaray D. P., Daware A., Maity S., Pattnaik S., and Bhattacharjee S. - Bioconversion of fish solid waste into PHB using Bacillussubtilis based submerged fermentation process, Environmental Technology **38** (24) (2017) 3201-3208.

9. Hassan M. A., Bakhiet E. K., Hussein H. R., Ali S. G. - Statistical optimization studies for polyhydroxybutyrate (PHB) production by novel *Bacillus subtilis* using agricultural and industrial wastes,International Journal of Environmental Science and Technology **16** (7) (2018) 3497-3512.
10. Rodríguez-Contreras A., Koller M., Miranda-de Sousa Dias M., Calafell-Monfort M., Braunegg G., and Marqués-Calvo M. S. - High production of poly(3-hydroxybutyrate) from a wildBacillus megateriumBolivian strain, Journal of Applied Microbiology **114** (5) (2013) 1378-1387.
11. Law J. H. and Slepecky R. A. - Assay of poly-β-hydroxybutyric acid., J. Bacteriol **82** (1961) 33-36.
12. Rathika R., Janaki V., Shanthi K., andKamala-Kannan S. - Bioconversion of agro-industrial effluents for polyhydroxyalkanoates production using *Bacillus subtilis* RS1,International Journal of Environmental Science and Technology **16** (10) (2019) 5725-5734.
13. Baikar V., Rane A., Deopurkar R. - Characterization of polyhydroxyalkanoate produced by *Bacillus megaterium* VB89 isolated from Nisargruna biogas plant, Appl Bio chem Biotechnol **183** (1) (2017) 241-253.
14. Biglari N., Orita I., Fukui T., Sudesh K. - A study on the effects of increment and decrement repeated fed-batch feeding of glucose on the production of poly (3-hydroxybutyrate) [P (3HB)] by a newly engineered *Cupriavidus necator* NSDG-GG mutant in batch fill-and draw fermentation, J Biotechnol **307** (2020) 77-86.
15. Mohanrasu K., Rao R. G. R., Dinesh G. H., Zhang K., Prakash G. S., Song D. P., Muniyasamy S., Pugazhendhi A., Jeyaraman J., Arun A. - Optimization of media components and culture conditions for polyhydroxyalkanoates production by *Bacillus megaterium*, Fuel. **271** (2020) 117522. doi:10.1016/j.fuel.2020.117522.
16. Penkhrue W., Jendrossek D., Khanongnuch C., Pathom-Aree W., Aizawa T., Behrens R. L., and Lumyong S. - Response surface method for polyhydroxybutyrate (PHB) bioplastic accumulation in *Bacillus drentensis* BP17 using pineapple peel, PloS one. **15**(3)(2020) e0230443. https://doi.org/10.1371/journal.pone.0230443
17. Vega-Castro O, Contreras-Calderon J., Leon E., Segura A., Arias M., Perez L., Sobral P. J. - Characterization of a polyhydroxyalkanoate obtained from pineapple peel waste using Ralsthoniaeutropha, J. Biotechnol. **231** (2016) 232-238.
18. Shamala T. R., Divyashree M. S., Davis R., Kumari K. L., Vijayendra S. V., Raj B. - Production and characterization of bacterial polyhydroxyalkanoate copolymers and evaluation of their blends by Fourier transform infrared spectroscopy and scanning electron microscopy, Indian J. Microbiol **49** (2009) 251-258.
19. Patel M., Gapes D. J., Newman R. H., Dare P. H. - Physico-chemical properties of polyhydroxybutyrate produced by mixed-culture nitrogen-fixing bacteria, Appl. Microbiol Biotechnol **82** (3) (2009) 545-555.
20. Jan S., Roblot C., Courtois J., Courtois B., Barbotin J. N., Séguin J. P. - 1H NMR spectroscopic dertermination of poly 3-hydroxybutyrate extracted from microbial biomass, Enzyme Microb Technol. **18** (3) (1996) 195-201.