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Bioconversion of waste cooking oil for the production of poly-3-hydroxybutyrate using *Bacillus cereus* MPTDC

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Used cooking oil is generated as a byproduct during frying process. It cannot be reused for cooking process due to health issues such as cancer and other digestive disorders. Alternative strategy is utilization of this waste cooking oil for production of poly-3-hydroxybutyrate (PHB) a biopolymer which can be used as a substitute for petroleum derived plastics or other value added products. In the present investigation, we used waste cooking oil as carbon source for PHB production by *Bacillus cereus* MPTDC. The optimum conditions of PHB production by *Bacillus cereus* MPTDC were waste cooking oil concentration of 2% (v/v), incubation time of 96 h, ammonium sulphate concentration of 7.5% and yeast extract concentration of 0.2%. Under optimized conditions the strain produced 3.777 g/L of PHB. The results indicate the potential of used cooking oil as carbon source for PHB production by *Bacillus cereus* MPTDC.

Keywords: Biodegradation, Biopolymer, Fermentation, Food Industry, Media Engineering, Optimization

Increasing environmental concerns and ecological issues raised by synthetic polymers necessitates the search for alternative biodegradable plastics. Poly-3-hydroxybutyrate (PHB) is a natural biopolymer produced by bacteria as an intracellular storage material when there is a nutrient limitation like nitrogen or phosphorous and the medium should contain an excess amount of carbon[1]. PHB are reported to be produced by different strains of *Bacillus*, *Pseudomonas*, *Alcaligenes*, *Cupriavidus*, etc.[1] PHB is completely degraded by a wide variety of microorganisms into CO_2 and water[1]. While, the petroleum derived plastics takes several years for partial degradation.

One of the major challenges in commercialization of PHB is the cost associated with the carbon source. This contributes to more than 50% of the overall production costs[1]. To develop an economically viable strategy for PHB production has been a challenge for researchers across the globe. Another limitation for commercialization of PHB is the instabilities of their molecular weight and structure which in turn affects the thermomechanical properties. The low extension and break and brittleness could be overcome by blending PHB with other polymers like starch, cellulose, polylactic acid, etc. Blending improves the properties of PHB over pure PHB. Most of the extraction and purification strategies adopted for PHB involve usage of organosolvents, which are expensive as well as create a lot of environmental issues. Several researches are going on to develop an economically viable and ecofriendly strategy for extraction of PHB.

One of the strategies adopted for development of an economically viable process is utilization of waste resources as a feed stock for production of PHB. Several waste streams like biodiesel industry generated crude glycerol[1], used cooking oil[2], pentose stream from pretreated biomass[3], hydrolyzate obtained from enzymatic saccharification of biomass[4], molasses[5], food waste[6], cafeteria waste[7]. etc., are reported. Utilization of the waste resources addresses

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two issues like waste management and conversion to an ecofriendly biopolymer.

Waste cooking oil generated during frying process is creating lots of health as well as ecological issues. Utilization of the waste cooking oil for animal feed is banned due to presence of harmful compounds generated during frying process[8]. An alternative strategy is to utilize this waste cooking oil as a suitable feed stock for production of PHB by microorganisms or can be used for biodiesel production is reported by Panadare & Rathod[9]. PHB production using waste cooking oil has been reported by several researchers[10-12]. Most of the industries producing PHB at commercial scale adopt two-stage fermentation. In the first stage, bacterial cells are grown in a medium to get sufficient biomass. In the second stage, the cells were subjected to a nutrient limited medium for production of biopolymer.

In the present study, we have attempted isolation of potential microbes from sewage and effluent samples for production of PHB and did media engineering to enhance PHB production by the most potent isolate using waste cooking oil as sole carbon source.

Materials and Methods

Sample

Sewage and effluent samples were collected from different parts of Kerala, India. Samples were stored in cold room till they are used for screening. Dilution plate technique was adopted for isolation of bacterial strains from sewage and effluent samples. Luria Bertani agar plates were used and incubated at 37°C for 48 h. Bacterial cultures were isolated and stock cultures were prepared. Subculturing was done for maintenance of the culture.

Primary screening of microbes for PHB production

All isolates were screened for PHB production by adopting Nile red staining[13]. Pre-inoculum was prepared by growing the cultures in a LB media (HiMedia, India) at 37°C for 24 h.PHB production was carried out in Nitrogen limited media as per the protocol adopted by Sindhu *et al*.,[14]. Positive isolates for PHB production will show an orange red fluorescence after Nile red staining and observing under fluorescent microscope.

Secondary screening

All the positive isolates were checked for the PHB yield by adopting HPLC analysis as per the protocol adopted by Law & Slepecky[15]. Inoculum concentration of 1.0% contains 4×10^8 CFU/mL. The estimation of PHB was carried out using HPLC. 0.2 g of lyophilized (Freeze dried at −108°C) biomass was taken and 1.0 mL of concentrated H_2SO_4 (SRL, India) was added. It was then incubated at 90°C for 30 min. To the sample, 4 mL of 0.01 N H_2SO_4 (SRL, India) was added. From this, 200 µL of sample was taken and 800 µL of 0.01 N H_2SO_4 (SRL, India) was added. The samples were filtered with 0.2 µm filters. The samples were injected into HPLC instrument (Prominence UFLC-XR, Shimadzu Co, Japan). The PHB analysis by HPLC after hydrolysis of the polymer was used as technique of titration of the PHB in aqueous environment. The monomeric essential units are obtained by exposure in concentrated H_2SO_4. The acid hydrolysis is accelerated by the heat. The polymer is at the same time hydrolyzed and dehydrated giving crotonic acid (CA) as product of reaction.

PHB standards were prepared by taking 1.0, 1.5, 2.0, 2.5, 3.0 mg of PHB and added 1.0 mL of concentrated H_2SO_4, followed by incubation at 90°C for 30 min. After cooling, added 4 mL of 0.01N H_2SO_4. Filter the standard samples, the total PHB yield can be calculated.

Identification of microbes and phylogenetic analysis

Identification of Bacteria

A sufficiently grown bacterial cell was pelleted at 10000×g for 1.0 min (Sigma, Germany). The bacterial genomic DNA was isolated using DNA isolation from Sigma (Germany). The 16S rRNA gene fragment was amplified using the universal primers, 27F, 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1525R, 5′-AAGGAGGTGATCCAGCC-3′. The PCR product was purified and the gene sequencing was carried out using the dye termination method. The sequenced 16S rRNA genomic sequences were analyzed using nucleotide blast search with the GenBank sequences

Phylogenetic analysis

The deduced 16S genomic DNA sequences were searched against Genbank deposited sequences of NCBI using BLAST. The sequences obtained with close similarity were used for multiple alignments and phylogenetic analysis were done using MEGA version 7.0 program.

Media engineering for PHB production

Plackett-Burman design was adopted to select the significant parameters affecting PHB production using waste cooking oil collected from CSIR-NIIST

canteen as sole carbon source. The parameters selected include pH, incubation time, ammonium sulphate concentration, yeast extract concentration, waste cooking oil concentration and inoculums concentration. These parameters were selected at lower and higher levels. The experiment involves 12 runs.

Significant parameters from the Plackett-Burman design were further optimized by adopting Box-Behnken design. The selected parameters include ammonium sulphate concentration, yeast extract concentration and inoculums concentration. These parameters were selected at lower, middle and higher levels. The experiment involves 15 runs.

Results and Discussion

Primary and secondary screening profile of isolates for PHB production

All the isolates were screened for PHB production by Nile red staining. Six positive isolates were obtained after primary screening. Positive cultures exhibited an orange red fluorescence. Results were depicted in Fig. 1. Primary screening will provide an idea whether the isolated strain is PHB producer or not.

Secondary screening will provide the actual PHB production profile of positive cultures. It was done to select the best strain based on the PHB yield. HPLC analysis was carried out for quantitative analysis of PHB. Details are depicted in Fig. 2.

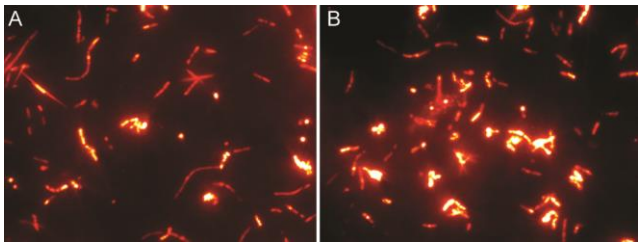

Fig.1 — Positive cultures showing orange red fluorescence on Nile red staining

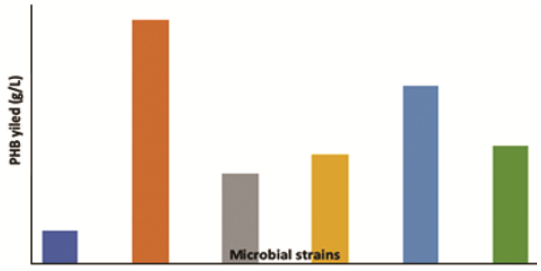

Fig.2 — Secondary screening profile of microbes for poly-3-hydroxybutyrate production

Bacterial identification and Phylogenetic analysis

The 16S rRNA gene was isolated by PCR and bacterial identification of the highly potent strain was identified by the 16S rRNA sequencing (1286 bp) and the sequence elucidated in this work was deposited in Gen Bank with accession number MK392133. Nucleotide BLAST analysis and phylogenetic analysis depicted that the 16S rRNA nucleotide sequences of MPTDC showed highly similarity (99%) to the16S rRNA sequences of *Bacillus cereus* (Gen Bank accession no. MN749822.1). Phylogenetic tree was constructed based on 16S rRNA sequences using neighbour-joining method for the isolate, MPTDC (Fig. 3).

Media engineering for PHB production using *Bacillus cereus* MPTDC

The results of Plackett-Burman design are depicted in Table 1. The results indicates that maximum PHB production was observed with Run number 3

Table 1 — Plackett-Burman design for poly-3-hydroxybutyrate production using used cooking oil as sole carbon source

Run no:	pH	Incubation time (h)	Ammonium sulphate (g/L)	Yeast extract (g/L)	Used cooking oil (%)	Inoculum conc. (%)	PHB yield (g/L)
1	7	96	0.1	0.5	3	3	0.444
2	5	96	0.8	0.1	8	3	0.333
3	5	96	0.1	0.1	3	6	1.277
4	5	72	0.1	0.5	8	6	0.321
5	7	72	0.8	0.5	3	6	0.195
6	7	96	0.8	0.1	8	6	0.201
7	7	72	0.8	0.1	3	3	0.072
8	5	72	0.8	0.5	8	3	0.190
9	7	96	0.1	0.5	8	3	0.711
10	5	96	0.8	0.5	3	6	0.463
11	5	72	0.1	0.1	3	3	0.545
12	7	72	0.1	0.1	8	6	1.079

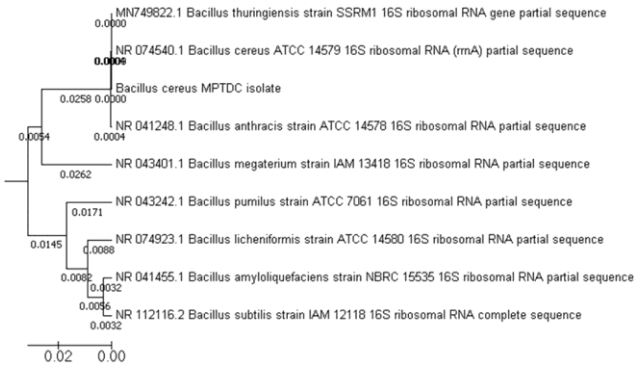

Fig. 3 —Phylogenetic tree developed based on 16S rRNA sequences using MEGA7 for the isolate, MPTDC and the GenBank sequences. [Branch distances represent nucleotide substitution rate and scale bar represents the number of changes per nucleotide position]

(1.277 g/L), where the conditions are pH of 5.0, incubation time of 96 h, waste cooking oil concentration of 3%,$(NH)_2SO_4$ concentration of 0.1%, yeast extract concentration of 0.1% and inoculums concentration of 6%.

Verlinden *et al*.[2] reported PHB production by *Cupriavidus necator* using waste frying oil. The strain produced 1.2 g/L of PHB which is comparable to PHB production using glucose. Higher PHB productions using various oils were reported for *C. necator* by del Rocio *et al*.[16]. Kamilah *et al*.[17] reported PHB production using palm oil based waste cooking oil by *C. necator* H16. The strain produced 60-80% of PHB and the cell dry weight was 14-17 g/L. In the present study, *Bacillus cereus* MPTDC also showed similar productivity like *Cupriavidus necator* using waste frying oil. Control experiments were carried out using pure cooking oil as sole carbon source. *Bacillus cereus* MPTDC produced more PHB yield (4.5 g/L) than using the used cooking oil as sole carbon source. This may be due to change in composition of the oil during heating. Pure vegetable oil contains mainly fatty acids[18]. Prolonged heating may cause oxidation of fatty acids which have an adverse effect on PHB production[19]. Here, we used waste cooking oil as such without any pretreatment. The pretreatment step was eliminated since it will increase the overall process economics.

In this study, waste cooking oil of 3% gave the highest PHB yield by *Bacillus cereus* MPTDC (1.277 g/L). Kongpeng *et al*.[20] observed the highest PHB production using 30 g/L concentration of used cooking oil by *C. necator* H16. The strain produced 27.36% of PHB content. Parameters, such as incubation time and inoculums concentration have a positive impact on PHB production, while yeast extract and ammonium sulphate concentration have a negative impact on PHB production by *B. cereus* MPTDC. Details are presented in Fig. 4.

The results of Box-Behnken design is depicted in Table 2. The results indicates that maximum PHB production(3.777 g/L) was observed with Run number 14, where the conditions are ammonium sulphate concentration of 0.15%, inoculum concentration of 7.5% and yeast extract concentration of 0.2%.

P value determines the significance of each of the coefficients. The value less than 0.95 are significant. In the present study, yeast extract is the one and only significant parameter. R^2 value is 99.33, indicating that the model can explain 99.33% variability on response. Details are depicted in Table 3.

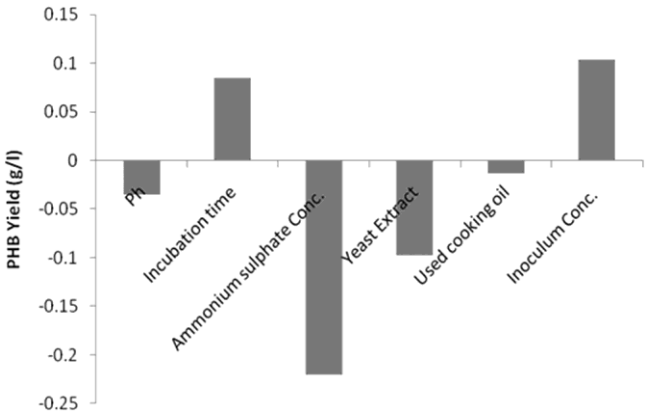

Fig.4 — Pareto chart depicting effect of different process parameters on poly-3-hydroxybutyrate production by microbial consortium using waste cooking oil as sole carbon source

Table 2 — Box-Behnken design for PHB production using waste cooking oil as sole carbon source

Run no:	Ammonium sulphate (%)	Yeast extract (%)	Inoculum conc. (%)	PHB yield (g/L)
1	0.15	0.2	7.5	3.675
2	0.15	0.3	9	2.443
3	0.2	0.2	9	2.529
4	0.1	0.2	6	2.532
5	0.15	0.1	9	2.53
6	0.15	0.3	6	2.177
7	0.15	0.1	6	2.77
8	0.1	0.3	7.5	2.84
9	0.1	0.2	9	2.767
10	0.15	0.2	7.5	3.678
11	0.1	0.1	7.5	2.259
12	0.2	0.2	6	2.537
13	0.2	0.3	7.5	2.117
14	0.15	0.2	7.5	3.777
15	0.2	0.1	7.5	3.101

Table 3 — Analysis of variance.
Estimated regression coefficients for PHB yield (g/L)

Term	Coef	SE Coef	T	P
Constant	3.77667	0.04482	84.261	0.000
Ammonium sulphate	–0.01425	0.02745	–0.519	0.626
YE	–0.13538	0.02745	–4.932	0.004
Inoculum conc.	0.03162	0.02745	1.152	0.301
Ammonium sulphate* Ammonium sulphate	–0.54308	0.04040	–13.442	0.000
YE*YE	–0.65433	0.04040	–16.196	0.000
Inoculum conc.* Inoculum conc.	–0.64233	0.04040	–15.899	0.000
Ammonium sulphate* YE	–0.39125	0.03882	–10.080	0.000
Ammonium sulphate* Inoculum conc.	–0.06075	0.03882	–1.565	0.178
YE[8]Inoculum conc.	0.12650	0.03882	3.259	0.022

[S = 0.0776324, PRESS = 0.482079;
R-Sq = 99.33%; R-Sq (pred) = 89.33%; and R-Sq(adj) = 98.13%]

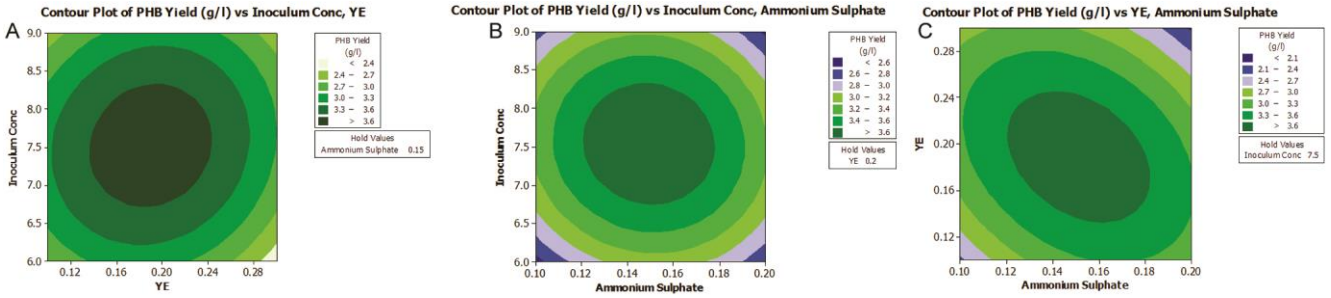

Fig. 5 — Contour plots showing interactions between different process parameters affecting poly-3-hydroxybutyrate production by microbial consortium using waste cooking oil as sole carbon source. (A) interactions between inoculums concentration and yeast extract; (B) interactions between inoculums concentration and ammonium sulphate concentration; and (C) interactions between yeast extract and ammonium sulphate concentration.

The PHB yield is determined as follows:

PHB yield (g/L) = $3.77667 - 0.01425X_1 - 0.13538X_2 + 0.0136X_3^2 - 0.54308X_1^2 - 0.65433X_2^2 - 0.64233X_3^2 - 0.39125X_1X_2 - 0.06075X_1X_3 + 0.12650X_2X_3$

where X_1, X_2 and $X_3$are $(NH_4)_2SO_4$ concentration, yeast extract concentration and inoculum concentration, respectively.

Contour plots showing interactions between different process parameters affecting PHB production by *Bacillus cereus* MPTDC using waste cooking oil as sole carbon source is depicted in Fig. 5 A-C. Fig. 5A shows interaction between inoculums concentration and yeast extract concentration on PHB yield. At low levels of inoculums concentration (6.0–7.0%) and yeast extract concentration (0.12–0.16%) the PHB yield is low (2.7 g/L); it increases with increase of inoculums concentration and yeast extract concentration. Maximum PHB production (3.6 g/L) was observed with high levels of inoculums concentration (7.0–8.0%) and middle levels of yeast extract concentration (0.16–0.24 %).

Figure 5B shows interactions between inoculum concentration and ammonium sulphate concentration on PHB yield. At low to middle levels of inoculums concentration (6.0–7.0%) and ammonium sulphate concentration (0.1–0.13%) the PHB yield is low (3.0 g/L); it increases with increase of inoculums concentration and yeast extract concentration. Maximum PHB production (3.6 g/L) was observed with high levels of inoculums concentration (7.0–8.0%) and high levels of ammonium sulphate concentration (0.13–0.17%). Gomma[5] observed yeast extract as best nitrogen source and inoculum concentration of 8% for maximum PHB yield by *Bacillus subtilis*.

Figure 5C shows interactions between yeast extract concentration and ammonium sulphate concentration on PHB yield. At low to high levels of yeast extract concentration (0.1–0.13%) and low levels of ammonium sulphate concentration (0.1–0.13 %) gave same PHB yield (4.5 g/L). Maximum PHB yield (3.6 g/L) was observed at middle levels of ammonium sulphate concentration (0.14–0.18 g/L) and middle levels of yeast extract concentration (0.14–0.24 %). Ammonium salts serves as a good source for the production of biomass for accumulation of PHB[21].

Conclusion

The present study indicates the potential of used cooking oil for production of poly-3-hydroxybutyrate (PHB) by *Bacillus cereus* MPTDC. This is the first report on PHB production by *B. cereus* MPTDC using used cooking oil. Under optimized conditions, the strain produced 3.777 g/L of PHB. The optimum conditions of PHB production using waste cooking oil by *B. cereus* MPTDC are waste cooking oil concentration of 2% (v/v), incubation time of 96 h, ammonium sulphate concentration of 0.15%, inoculum concentration of 7.5% and yeast extract concentration of 0.2%. Media engineering as well strain improvement could improve the PHB yield. Targeting multiple value added products should make the process commercially viable.

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Conflict of Interest

Authors declare no conflict of interests.

References

1 Jincy M, Sindhu R, Pandey A & Binod P, Development of a bioprocess strategy for utilizing biodiesel industry generated crude glycerol for the production of poly-3-hydroxybutyrate. *J Sci Ind Res*, 72 (2013) 596.

2 Verlinden RAJ, Hill DJ, Kenward MA, Williams CD, Piotrowska-Seget Z & Radecka IK, Production of poly-hydroxyalkanoates from waste frying oil by *Cupriavidus necator AMB Express*, 1 (2011) 11.

3 Sindhu R, Silviya N, Binod P & Pandey A, Pentose rich hydrolysate from acid pretreated rice straw as a carbon source for the production of poly-3-hydroxybutyrate. *Biochem Eng J*, 78 (2013) 67.

4 Prabisha TP, Sindhu R, Binod P, Sajitha S & Pandey A, Alkali pretreated sugarcane tops hydrolysate for the production of poly -3-hydroxybutyrate by a dairy effluent isolate *Comomonas* sp. *Indian J Biotechnol*, 13 (2014) 306.

5 Gomaa EZ. Production of polyhydroxyalkanoates (PHAs) by *Bacillus subtilis* and *Escherichia coli* grown on cane molasses fortified with ethanol. *Braz Arch Biol Technol*, 57 (2014) 145.

6 Sindhu R, Manju A, Mohan P, Rajesh RO, Madhavan A, Arun KB, Hazeena SH, Mohandas A, Rajamani SP, Puthiyamadam A, Binod P & Reshmy R, Valorization of food and kitchen waste: An integrated strategy adopted for the production of poly-3-hydroxybutyrate, bioethanol, pectinase and 2,3-butanediol. *Bioresour Technol*, 310 (2020) 123515.

7 Din MFMd, Huey SC, Yunus SM, Ahmad MA, Ponraj M, Ujang Z & Shreesivadasan C, Raw material resource for biodegradable plastic production from cafeteria waste. *J Sci Ind Res*, 71 (2012) 573.

8 Kulakrni MG & Dalai AK, Waste cooking oil an economic source for biodiesel: a review. *J Ind Engg Chem*, 45 (2006) 2901.

9 Panadare DC & Rathode VK, Applications of waste cooking oil other than biodiesel; a review. *Iranian J Chem Engg*, 13 (2015) 55.

10 Song JH, Che OJ, Mun HC, Shung, CY & Woojun P, Polyhydroxyalkaonate (PHA) production using waste cooking oil by *Pseudomonas* sp. strain DR2. *J Microbiol Biotechnol*, 18 (2009) 1408.

11 Tsuge T, Salto Y, Kikkawa Y, Hiraishi T & Doi Y, Biosynthesis and compositional regulation of poly-[(3-hydroxybutyrate)-co-(3-hydroxyexaonate)] in recombinant *Ralstonia eutropha* expressing mutated polyhydroxyalkoanate synthase genes. *Macromolecular Biosci*, 4 (2004) 238.

12 Rao U, Sridhar R & Sehgal PK, Biosynthesis and biocompatibility of poly (3-hydroxybutyrate –co-4-hydroxybutyrate) produced by *Cupriavidus necator* from spent palm oil. *Biochem Engg J*, 49 (2010) 13.

13 Chen W, Zhang C, Song L, Sommerfeld M & Hu Q, A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *J Microbiol Meth*, 77 (2009) 41.

14 Sindhu R, Ammu B, Binod P, Deepthi KS, Ramachandran KB, Soccol CR & Pandey A, Production and characterization of poly-3- hydroxybutyrate from crude glycerol by *Bacillus sphaericus* NII 0838 and improving its thermal properties by blending with other polymers.*Braz Arch Biol Technol*, 54 (2011) 783.

15 Law JH & Slepecky RA, Assay of poly-β-hydroxybutyric acid. *J Bacteriol*, 82 (1961) 33.

16 del Rocio LCM, Noel GRJ & Fermin PG, Production of polyhydroxyalcanoates by *Wautersia eutropha* using vegetable oils as carbon source. *J Biotechnol*, 131 (2007) S156.

17 Kamilah H, Al-Gheethi A, Yang TA & Sudesh K, The use of palm-oil based waste cooking oil to enhance the production of poly-3-hydroxybutyrate [P{3HB}] by *Cupriavidus necator* H16 strain. *Arabian J Sci Engg*, 43 (2018) 3453.

18 Tabee E, *Vegetable oils and fried potato products*. (PhD thesis, Swedish University of Agricultural Sciences, Uppsala), 2008.

19 Boskou D, Frying temperatures and minor constituents of oils and fats. *Grasas y Aceites*, 49 (1998) 326.

20 Kongpeng C, Iewkittayakorn J & Chotigeat W, Effect of storage time and concentration of used cooking oil on polyhydroxyalkanoates (phas) production by *Cupriavidus necator* H16. *Sains Malaysiana*, 46 (2017) 1465.

21 Beaulieu M, Beaulieu Y, Melinard J, Pandian S & Goulet J, Influence of ammonium salts and cane molasses on growth of *Alcaligenes eutrophus* and production of polyhydroxybutyrate. *Appl Environ Microbiol*, 61 (1995) 165.