Italian Journal of Food Science, 2022; 34 (3): 48–58

Valorisation of date fruits by-products for the production of biopolymer polyhydroxybutyrate (PHB) using the bacterial strain *Bacillus paramycoides*

Zohra Chekroud[1]*, Leila Djerrab[2], Amer Rouabhia[3], Mohamed Abdesselem Dems[4], Imane Atailia[5], Faiçal Djazy[2], Mustapha Adnane Smadi[6]

[1]Research Laboratory of Interactions, Biodiversity, Ecosystems and Biotechnology, LRIBEB University, Skikda, Algeria; [2]Laboratory of Research on the Physical Chemistry of Surfaces and Interfaces LRPCSI University, Skikda, Algeria; [3]Research Laboratory of Biological Treatment of Residues, Department of Chemical Engineering and Food Technology, Institute of Vitivinicultural and Agri-food Research (IVAGRO), University of Cadiz, Cadiz, Spain; [4]Bioinformatics and Biostatistics Unit (BIBSU), National Center for Biotechnology, Constantine, Algeria; [5]Superior School of Food Sciences and Agro Food Industries, El Harrach, Algeria; [6]Laboratory of Animals Biotechnologies, National Center for Biotechnology, Constantine, Algeria

***Corresponding Author**: Zohra Chekroud, Research Laboratory of Interactions, Biodiversity, Ecosystems and Biotechnology LRIBEB. University of 20th August 1955-Skikda. B.P.26, El Hadeik road 2100 Skikda, Algeria. Email: chekroudzohra@yahoo.fr

Received: 20 May 2022; Accepted: 27 July 2022; Published: 23 August 2022

PAPER

Abstract

The aim of this research was to study the valorization of the date fruit by-product by conversion of date syrup into biopolymer polyhydroxybutyrate (PHB), based on the metabolic capacity of the bacterial strain *Bacillus paramycoides* to accumulate PHB from date syrup. Total reducing sugars in date syrup was essayed using 3',5-dinitrosalicylic acid (DNS) and HPLC methods. *Bacillus paramycoides* was isolated from soil of the botanic garden of Skikda university, Algeria. The accumulated PHB was extracted using chloroform. It was quantified as crotonic acid in concentrated sulfuric acid (H_2SO_4) by spectrophotometry at 300nm. Date syrup is characterized by high levels of total sugars (79.66 g/L) with 31.86 g/L of total reducing sugars. PHB accumulation reached its maximum (104.3 ug/mL) after 96 h of incubation at pH 7 and temperature 37°C using Tryptophane as the nitrogen source and acid pretreated syrup at a concentration of 8%. HPLC analysis on Aminex HPX-87H showed that the produced PHB from date syrup is characterized by a chromatogram peak with a retention time at 22.5 min.

Keywords: *Bacillus paramycoides*; bioprocess; date syrup; Polyhydroxybutyrate (PHB); valorization

Introduction

Date palm *Phoenix dactylifera* is a tropical and subtropical tree (Chandrasekaran and Bahkali, 2013). It is the main crop of Algerian Saharan agriculture with an average production estimated at 420.290 million tons (Bouguedoura *et al*., 2015). This production is however accompanied by a substantial loss of large amounts of date during the postharvest processes (Abbés *et al*., 2011; Nancib *et al*., 2015). Due to their soft texture, the lost dates known as date by-products are not edible and are often discarded (Chandrasekaran and Bahkali, 2013). They are mainly used for animal feed (Majzoobi *et al*., 2020). Fermentation processes employing microoganisms is the most common

ISSN 1120-1770 online, DOI 10.15586/ijfs.v34i3.2236

method for biosynthesis of value products using date products and wastes as raw materials. Many eco-friendly products could be derived from date by-products such as organic acids, enzymes, amino acids, biomass (Chandrasekaran and Bahkali, 2013), bioethanol (Ahmad *et al*., 2021), and biopolymers. Omar *et al*. (2001) used date molasses for the production of Polyhydroxybutyrate (PHB) using the bacterial strain *Bacillus megaterium*.

Hence, petrochemical polymers led to severe crisis of the environment with detrimental impact on the ecosystems (Mohapatra *et al*., 2020), and there has been an increasing demand to produce eco-friendly biopolymers synthesized by microbes and plants from inexpensive and renewable sources (Narayanan *et al*., 2020) like agro-food wastes. PHB has attracted much attention in recent years due to its varied properties (thermoplastic and elastomeric), biocompatibility, and biodegradability (Keshavarz and Roy, 2010). It was reported that PHB could be synthesized by many Gram-positive and Gram-negative bacteria as intracellular carbon and energy reserve material under nitrogen and phosphorus limiting conditions and surplus of carbon source (Anderson and Dawes, 1990). The main constraint in PHB production is accounted to the cost of raw materials, thus the use of cheap carbon sources like agro food wastes cloud be highly significant (Singh *et al*., 2013).

The goal of this research was to study a bioprocess technology for the valorization of date fruit by-products to a biodegradable biopolymer and to provide an alternative solution for non renewable fossil resources. The bioprocess is based on the capacity of the bacterial strain *Bacillus paramycoides* to accumulate PHB from date syrup.

Material and methods

Preparation of date syrup

Date fruits of the variety "Deglet Nour" with poor commercial quality were collected during the month of September 2020, from a private factory in the South of Algeria specialized in the exportation of dates. The fruits were pitted, washed with distilled water, and cut into small pieces. They were added to hot water at a ratio of 1/2.5 (weight/volume) (Chniti *et al*., 2017). The obtained juice was filtered through a gauze and hand pressed. The juice was then boiled at 70°C for 30 min (El-Nagga and Abd El-tawab, 2012) until obtaining a concentrated thick, dark syrup. The final crude syrup was stored in sterilized dark bottles at room temperature.

Treatment of date syrup

The obtained crude syrup underwent two types of treatment: centrifugation and hydrolytic treatment. A quantity of 15g of syrup was weighed and diluted in 100 mL of sterile distilled water (Ashraf *et al*., 2015). The obtained solution was centrifuged at 3500 g for 15 min and the supernatant was recovered and filtered using Whatman filter paper no.1. For hydrolytic treatment, the obtained filtered supernatant was hydrolyzed by adding 5 mL of 5M or 1.5 N sulfuric acid or 3M HCl to 100 mL of the solution. The whole concoction was incubated at 90°C in water bath for 1h. Date syrup was then centrifuged at 3000 g for 15 min (Kundu *et al*., 1984) to eliminate the formed debris. Finally, pH was adjusted to 7 using 1M NaOH. At the end of the treatment two phases were formed; a clear black supernatant and a brown pellet. The supernatant was recovered to be used later. Crude, centrifuged, and acid hydrolyzed syrups were sterilized by tyndallization to avoid thermal degradation of sugars.

Physico-chemical characteristics of date syrup

pH, alkalinity, and total solids of the obtained syrup were determined according to standard methods (Tallon *et al*., 2005). pH was measured directly using a calibrated pH meter (Crison GLP21) after agitation of the sample. The alkalinity was determined by diluting 1mL of the sample into 50 mL of distilled water. The solution was then titrated by H_2SO_4 until the pH 4.3. Total solids were measured after drying the syrup at 110 ± 5°C. Total sugars (T Sug) with sucrose and reducing sugars were essayed by an HPLC equipped with a refractive index detector and a Shodex column (SH1011, 8.0 × 300 nm). Total reducing sugars (TRS) after date syrup acid treatment was essayed using 3',5-dinitrosalicylic acid (DNS) method (Gusakov *et al*., 2011). A volume of 25 uL of DNS was firstly added to 25 uL of the diluted sample (20 uL of sample with 925 uL distilled water). The obtained solution was heated for 10 min at 105°C and cooled for 5 min. Distilled water (250 uL) was added to the cold solution and absorbance was read at 540nm against a blank of distilled water. The concentration of reducing sugars was determined from a calibration curve previously prepared from different concentrations of glucose (0.25–5 g/L).

Primary screening of the bacterial strains producing PHB

The bacterial strain producing PHB from date syrup was isolated from the soil of the botanic garden of Skikda university, Algeria. Pure bacterial colonies isolated from 1 g of soil using the technique of serial dilutions were inoculated on mineral salt medium (MSM) agar medium (Sharma *et al*., 2007) added with 2% glucose as carbon source and incubated at 37°C for 48h. Colonies having the capacity to grow on the MSM agar medium with glucose were added to ethanolic solution with 3% Sudan Black B (C29H24N6 224-087 segma) for 30 min and those

giving dark blue color were considered as positive for PHB production (Mohd Zahari *et al*., 2012).

Secondary screening of the bacterial strains producing PHB

To select the best strain producing PHB, the colonies showing positive Sudan black staining were re-cultured in MSM liquid medium (Sharma *et al*., 2007) with 2% of glucose. A bacterial inoculum of each strain was prepared by inoculating a loop of the bacterial colony into 100 mL of nutrient broth in 250 mL conical flasks. The inoculated flasks were incubated for 24 h at 37°C with an agitation rate of 150rpm. Cells were then collected by centrifugation at 10,000 g for 15 min at 4°C, washed aseptically by sterile distilled water, and resuspended in 250 mL Erlenmeyer flasks containing 100 mL of liquid MSM mineral medium added with 2% glucose and incubated at 37°C for 72h. The strain showing the best yield of PHB accumulation was chosen to test its capacity to accumulate PHB using 2% of centrifuged date syrup instead of 2% glucose in MSM solid medium. Sudan black staining was used to confirm PHB production from date syrup.

The produced PHB bacterial strain from date syrup was then identified on the basis of its 16srRNA partial sequence by comparing consensus sequences to a database library of known 16srRNA gene sequences in GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) by multiple sequence alignment.

Optimization of physico-chemical PHB production conditions

To test the effect of physico-chemical factors on PHB production and bacterial cell growth, a bacterial inoculum was firstly prepared as previously described. Bacterial cells were recovered by centrifugation at 10,000 g for 15 min at 4°C and washed aseptically with sterile distilled water. They were resuspended in MSM liquid medium with 2% of centrifuged date syrup. pH of the medium was adjusted to 7 and the cultures were incubated in a rotary incubator at 37°C and an agitation rate of 150rpm during 24, 48, 72, and 96 h. The effect of aeration was tested by augmenting the agitation rate from 150 rpm to 200 rpm and 300 rpm. The pH was tested at 3, 7, and 8 by adding NaOH (2N) or HCl (2N). The cells growth and bioaccumulation of PHB from date syrup were also tested at 30°C, 37°C, and 44°C. The nitrogen source in the MSM medium (NH_4Cl) was substituted by ammonium sulfate, yeast extract, beef extract, and peptone, one at a time. The effect of syrup treatment was tested by replacing centrifuged date syrup with crude syrup and acid hydrolyzed syrup with 5M and 1.5 N sulfuric acid or with 3M HCl. Finally, the effect of syrup concentration was tested by adding 2, 4, 6, and 8% of 5M H_2SO_4 hydrolyzed date syrup to the medium, one at a time. The experiments were conducted in triplicates.

Extraction of PHB from bacterial cells

The technique of boiling chloroform was used. The bacterial cells accumulating PHB under different conditions were centrifuged at 4000 g for 10 min. The cells were resuspended in an equivalent amount of 4% NaCl and incubated for 1 h at 37°C. The cells pellet was washed with acetone, ethanol, and distilled sterile water to eliminate undesirable elements. The solution was recentrifuged and the supernatant was discarded. The polymer granules were dissolved in boiling chloroform, which was then allowed to evaporate (Adwitiya *et al*., 2009) to obtain pure PHB.

Quantification of PHB

The extracted PHB was quantified using an UV spectrophotometric analysis. The chloroform extracted PHB was converted to Crotonic acid. Ten milliliters of concentrated sulfuric acid (98%) was added to the chloroform dissolved PHB for 15 min. The solution was left for cooling. PHB was determined quantitatively as crotonic acid by measuring the absorbance at 300 nm in a UV spectrophotometer using H_2SO_4 solution as blank. Standard solution of pure crotonic acid was prepared at different concentrations (0.1–2.0 ug/mL). The quantity of accumulated PHB expressed as microgram per milliliter of bacterial cells (ug/mL) was measured by comparing the absorbance of PHB converted to crotonic acid with the absorbance of the standard pure crotonic acid concentrations (Elsayed *et al.*, 2013).

Cells growth

Ten milliliters of the culture medium containing bacterial cells accumulating PHB was centrifuged at 10,000 g for 10 min. The supernatant was discarded and the bacterial pellet was washed twice with sterile distilled water. The cells pellet was then scrubbed to a weighing pan and dried at 100°C for 48h. The dried cells were diluted to an appropriate concentration and the absorbance was measured at 600nm. Cells' dry matter was determined according to a standard curve (Naheed *et al*., 2012) previously generated from cells dry matter concentrations (0.1–1ug/mL). It was expressed as CDW ug/mL. The yield of PHB accumulation was calculated as percentage of PHB content (ug/mL) per cells dry matter (ug/mL).

Identification of PHB using Aminex HPX-85X. HPLC analysis

To confirm the synthesis of PHB polymers, samples containing PHB were analyzed by using an HPLC (LaChrom Elite VWR-Hitachi). Samples were eluted with 0.014 N H_2SO_4 at a flow rate of 0.7 mL min-1 from an Aminex HPX-87H ion exclusion organic acid analysis column (C18 4.6x250) (Torrance, CA, USA) preceded by an ion exclusion guard column of Aminex HPX-85X. HPLC. The produced PHB was measured as crotonic acid dissolved in concentrated H_2SO_4. Crotonic acid and pure PHB digested into crotonic acid using concentrated H_2SO_4 were used as standards.

Statistical analysis

All the experiments were conducted in triplicates. The results expressed as mean ± standard error were analyzed by one way ANOVA analysis of variance (one-way ANOVA), followed by pairwise comparisons using the Fisher's Least Significant Difference (LSD) post hoc test. The statistical significance was considered at $P < 0.05$. Data analysis was carried out using Statistica 10 software (StatSoft, Inc.).

Results

Date syrup characterization

Date syrup extracted from "Deglet Nour" was characterized by high levels of total sugars (T Sug) (79.66g/L) and a total reducing sugars (TRS) rate of 31.86 g/L (Table 1). TRS rate increased to 78.86 g/L,72.96g/L, and 72.2 g/L after treatment of date syrup with $5MH_2SO_4$, 3MHCL, and 1.5 M H_2SO_4, respectively (Table 1).

Screening of the bacterium producing PHB from date syrup

A total of 20 bacterial strains were isolated from the soil of the botanic garden of Skikda university. The preliminary screening of PHB producing strains was further identified by a Sudan black method. Nine isolates (BG1-BG9) showed a black-blue color when stained with Sudan Black B (Figure 1), which indicates that they are positive PHB producing. The quantitative screening in MSM liquid medium with 2% of glucose as carbon source revealed that the highest quantity of PHB was accumulated by the strain BG5 (95.67 ug/mL) followed by the strain BG2 (83.92 ug/mL) after 72h of incubation at 37°C (Figure 2). The strain showing the highest level of PHB accumulation was tested for its

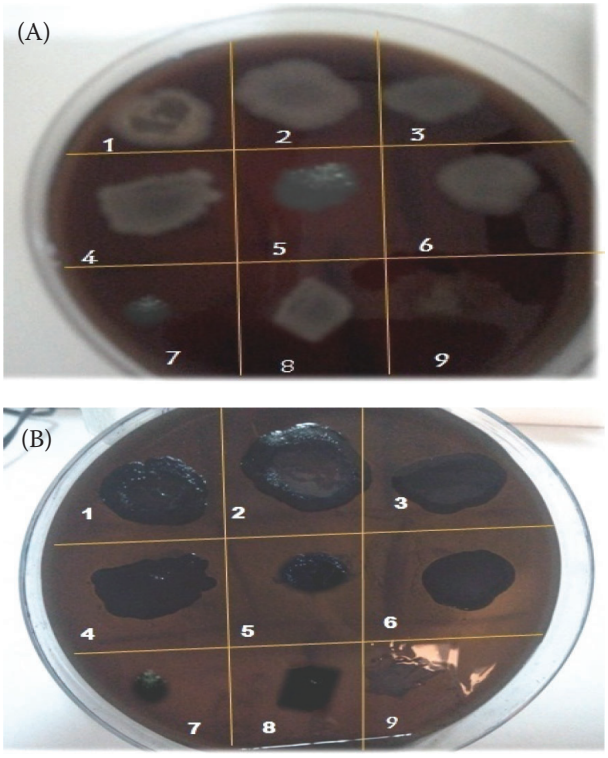

Figure 1. Screening of some bacterial isolates on MSM solid medium with date syrup as carbon source using Sudan Black staining. (A) Before staining, (B) After staining (green-blue colonies). 1:BG1; 2:BG2; 3:BG3; 4:BG4; 5:BG5; 6:BG6; 7:BG7;8:BG8; 9:BG9; BG1-BG9: the nine isolated bacterial strains showing positive PHB production using glucose as carbon source.

Table 1. Physico-chemical characteristics of date syrup.

	Parameters									
Date syrup	PH	TS(g/k)	TS%	Alkalinity g/L	T Sug content (g/L)	Sucrose content (g/L)	TRS content before date syrup acid treatment (g/L)	TRS content after date syrup acid treatment (g/L)		
								5M H_2SO_4	3M HCl	1.5 H_2SO_4
	5.63	713.8	79	15.8	79.66	47.8	31.86	78.86	72.96	72.2

TS: total solids; TS%: percentage of total solids; T Sug: total sugars; TRS: total reducing sugars before date syrup acid pretreatment; 5M H_2SO_4: total reducing sugars after date syrup pretreatment with 5M H_2SO_4; 3M HCL: total reducing sugars after date syrup pretreatment with 3M HCL, 1.5M H_2SO_4: total reducing sugars after date syrup pretreatment with 1.5 M H_2SO_4

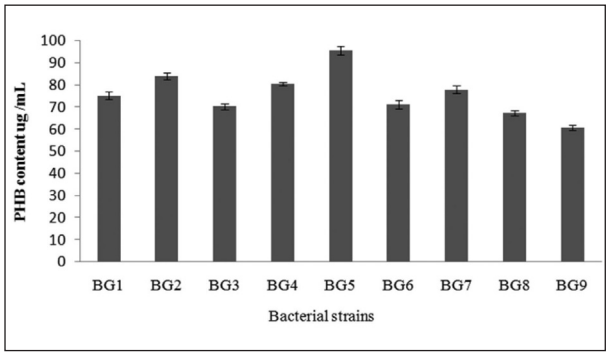

Figure 2. PHB content in the nine isolated bacterial strains. Results are expressed as mean of tri-replicates±standard error. BG1, BG2, BG3, BG4, BG5, BG6, BG7, BG8, and BG9: the nine isolated bacterial strains showing positive PHB productionusing glucose as carbon source.

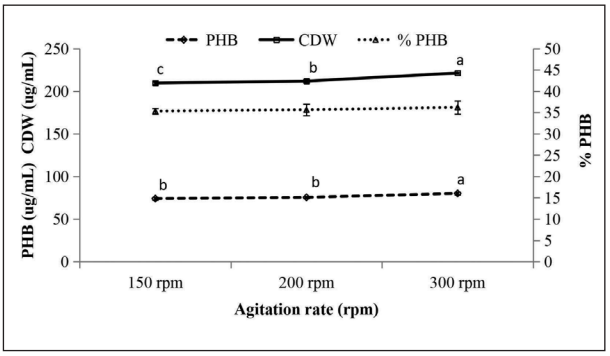

Figure 4. Effect of aeration on PHB accumulation. Results are calculated as mean of tri-triplicates ± standard error; different letters indicate significant differences using ANOVA test followed by the post-hoc Fisher test (LSD) at P < 0.05. P = 0.000 for PHB content, P = 0.001 for CDW.

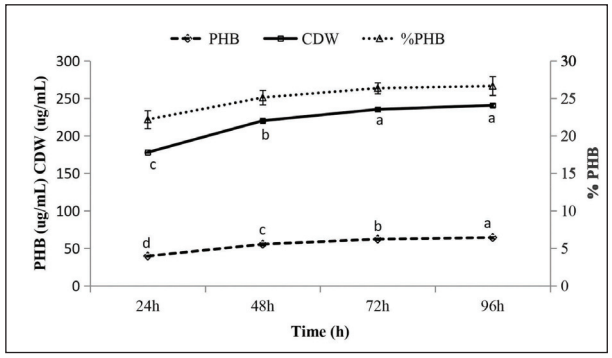

Figure 3. The effect of the incubation period on PHB accumulation. Results are calculated as mean of tri-triplicates ± standard error; different letters indicate significant differences using ANOVA test followed by the post-hoc Fisher test (LSD) at P < 0.05. P = 0.000 for both PHB and CDW.

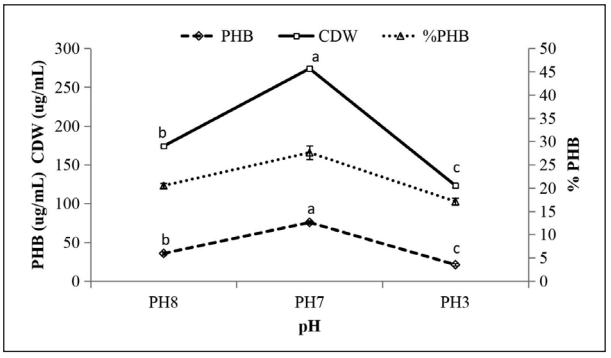

Figure 5. Effect of pH on PHB accumulation. Results are calculated as mean of tri-triplicates ± standard error; different letters indicate significant differences using ANOVA test followed by the post-hoc Fisher test (LSD) at P < 0.05. P = 0.0001 for both PHB content and CDW.

ability to accumulate PHB from date syrup instead of glucose. Sudan black staining confirmed the capacity of the strain BG5 to produce PHB from date syrup.

BG5 was identified on the basis of its partial 16srRNA sequencing by comparing consensus sequences to a database library of known 16srRNA gene sequences in GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Analysis showed high identity of BG5 with *Bacillus paramycoides* MCCC 1A04098, which has a partial 16s rRNA sequence NCBI *assessing number NR_157734.1.*

Effect of incubation period on PHB production

The results demonstrated in Figure 3 revealed that PHB yield was directly proportional with the incubation period. It increased gradually from 22.18% after 24h of incubation to 26.36% after 72h with a slight increase after 96h (26.65%), where it reached its maximum with a maximum PHB accumulation (64.14 ug/mL) and a maximum cell growth (240.69 ug/mL).

Effect of aeration rate

The aeration was monitored by agitation of the bacterial cultures. Our results (Figure 4) revealed that the PHB yield increased with the increase in the agitation rate from 150 rpm to 300 rpm. The maximum yield (36.22%) with a maximum PHB production (80.17 ug/mL) and a maximum cell growth (221.35 ug/mL) were recorded at 300 rpm.

Effect of pH

The rate of PHB production was tested in acid (pH 3), alkaline (pH 8), and neutral (pH 7) media. It was observed that the best rate of PHB accumulation (27.61%) was obtained at pH 7 with a PHB quantity of 75.66 ug/mL and a biomass of 274 ug/mL. It decreased significantly beyond pH 7 (Figure 5).

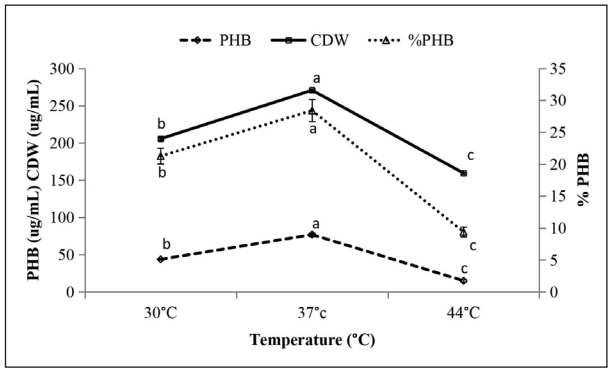

Figure 6. Effect of temperature on PHB accumulation. Results are calculated as mean of tri-triplicates ± standard error; different letters indicate significant differences using ANOVA test followed by the post-hoc Fisher test (LSD) at P < 0.05. P = 0.0001 for both PHB content and CDW.

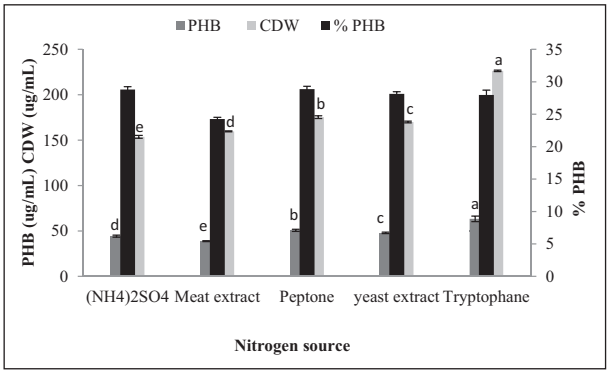

Figure 7. Effect of nitrogen sources on PHB accumulation. Results are calculated as mean of tri-triplicates ± standard error; different letters indicate significant differences using ANOVA test followed by the post-hoc Fisher test (LSD) at P < 0.05. P = 0.000 for both PHB content and CDW.

Effect of temperature

PHB synthesis increased from 21.67% at 30°C to 28.39% at 37°C. It decreased significantly at 44°C (9.40%). Maximum PHB quantity and maximum cell biomass were recorded at 37°C (76.85 ug/mL and 270.67 ug/mL, respectively) (Figure 6).

Effect of various nitrogen sources

Using tryptophane as a nitrogen source with date syrup gave the best PHB content (63.13 ug/mL) and cells biomass (226.33 ug/mL) followed by peptone (50.61 ug/mL PHB and 175.33 ug/mL CDW). There was, however, a decrease in cells biomass growth and PHB accumulation when we used inorganic nitrogen source $(NH_4)_2SO_4$ (44.25 ug/mL PHB and 153.67 ug/mL CDW) (Figure 7).

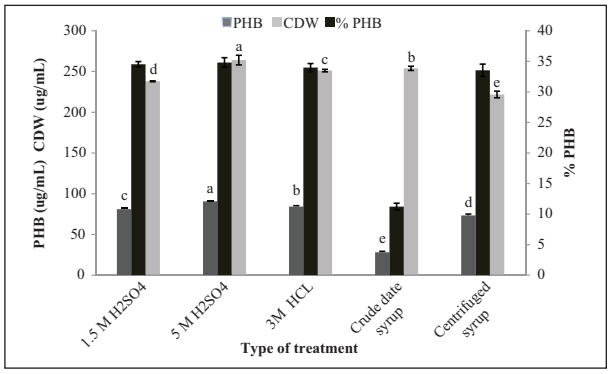

Figure 8. Effect of date syrup treatment on PHB accumulation. Results are calculated as mean of tri-triplicates ± standard error; different letters indicate significant differences using ANOVA test followed by the post-hoc Fisher test (LSD) at P < 0.05. P = 0.000 for both PHB content and CDW.

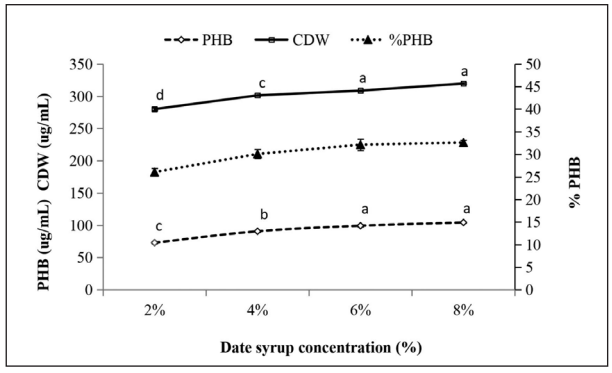

Figure 9. Effect of date syrup concentration on PHB accumulation. Results are calculated as mean of tri-triplicates ± standard error; different letters indicate significant differences using ANOVA test followed by the post-hoc Fisher test (LSD) at P < 0.05. P = 0.000 for both PHB content and CDW.

Effect of date syrup treatment

The effect of crude, centrifuged, and hydrolyzed date syrup was tested. It was noted that hydrolyzed date syrup with $5MH_2SO_4$ gave the best yield of PHB production (34.32%) with maximum PHB content (89.35 ug/mL) and maximum cells growth (260.33 ug/mL). There was however a significant decrease in PHB yield with crude syrup (11.06%) where the PHB content decreased to 27.68 ug/mL and the cells growth to 250.33 ug/mL (Figure 8).

Effect of date syrup concentration

The effect of syrup concentration on PHB accumulation by *Bacillus paramycoides* is demonstrated in Figure 9. The PHB yield was directly proportional with the concentration of date syrup. It increased

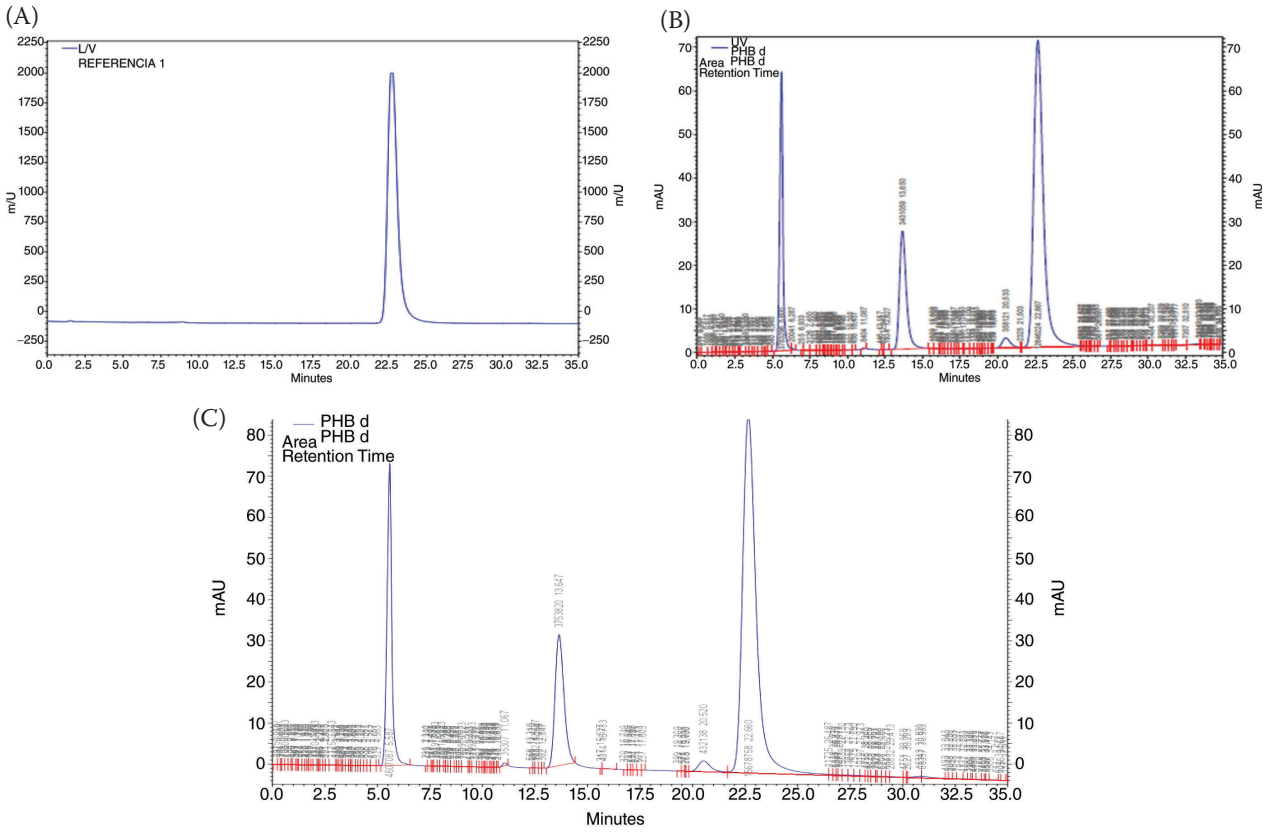

Figure 10. Aminex-HPLC analysis of PHB. (A) Standard crotonic acid (with retention time of 22.5min), (B) Standard pure PHB (with retention time of 22.5 min), (C) produced PHB from date syrup (with retention time of 22.5min).

from 26.06% at 2% of date syrup to 32.62% at 8%. The increase of PHB yield was accompanied with an increase of the PHB content and cell growth. We obtained 104.3 ug/mL of PHB and 319.73 ug/mL of cells' dry matter at 8% of syrup.

The physico-chemical conditions were monitored in triplicates. Results were expressed as mean± standard error. Variance analysis (ANOVA) showed that there was a very high significant difference between all the conditions ($Pr < 0.05$). Statistically, the multiple comparisons of the means performed with the LSD post hoc test indicated that the highest amount of PHB production was obtained after 96h of incubation at 37°C and pH 7, using tryptophane as nitrogen source and 8% of hydrolyzed syrup by 5M H_2SO_4 as the carbon source.

HPLC analysis of produced PHB

The PHB produced by *Bacillus paramycoides* using date syrup was identified by an HPLC on Aminex HPX-87H technique. The obtained chromatograms of re-crystallized crotonic acid (Figure 10A), pure PHB converted to crotonic acid (Figure 10B), and PHB produced by *Bacillus paramycoides* from date syrup converted to crotonic acid (Figure 10C) revealed that the three chromatograms share the same peak with a retention time of 22.5 min. This confirms the synthesis of PHB by *Bacillus paramycoides* using date syrup as the carbon source.

Discussion

The agro-food industry produces large amounts of wastes. Many strategies have been established in Europe and other countries of the world for the valorization of food waste streams and the recovery of biomolecules (Baiano, 2014). In this study, an isolated bacterial strain from the botanic garden of Skikda university (BG5) showing the best PHB accumulation using 2% of glucose (95.67 ug/mL) (Figure 2) was confirmed by Sudan black staining to accumulate PHB using 2% of date syrup, and it was identified as *Bacillus paramycoides.* Many bacterial strains have been confirmed to accumulate PHB from agro wastes like date syrup (Mostafa *et al.*, 2020), banana peel, sugarcane bagasse, corn cob, and teff (*Eragrostistef*) straw (Getachew and Woldesenbet, 2016). A wide range of PHB producer *Bacillus* species are recorded in the literature (Mohapatra *et al.,* 2017). *Bacillus pumilus*, *Bacillus megaterium*, and *Bacillus subtilis* have been reported to produce PHB from

agro-food wastes (Singh *et al*., 2013; Vu *et al*., 2021; Werlang *et al*., 2021). The bacterial aptitude for utilizing complex carbon sources like agro-food wastes varies according to the material biochemical composition and the enzymes involved by the bacterium (Belal, 2013). The date syrup used in our approach contains high levels of sugars (79.66 g/L) with 31.86 g/L of reducing sugars (Table 1), which makes it a promising source for PHB production since bacteria accumulates PHB under nutrient stress conditions and surplus of carbon source (Blunt *et al.*, 2018).

The optimization of fermentation parameters is among the strategies now used to improve PHB production (Gurieff and Lant, 2007). The yield of PHB accumulation reached its maximum after 96h of incubation (26.65%). The best incubation period for PHB accumulation depends on the bacterial strain. The increase in the PHB content (64.14 ug/mL) and cells growth (240.7 ug/mL) at this incubation period (Figure 3) is in accordance with the results recorded by Gomaa (2014) and Mostafa *et al.* (2020). Some authors however reported that the best incubation periods are 48h (Thapa *et al*., 2018) and 72 h (Singh *et al*., 2013).

Agitation is an important factor for PHB production. It helps in mixing the oxygen, the heat and the nutrients and facilitates the distribution of air in the nutrient broth so that it increases the liquid-gaz contact area (Mantzouridou *et al.*, 2002). This research revealed that increasing the agitation rate to 300 rpm increased the cells growth (221.35 ug/mL) and the PHB content (80.17 ug/mL) (Figure 4). Slow agitation rate leads to an increase in the viscosity of the culture medium and a reduction of the mass transfer (Bandaiphet and Prasertsan, 2006).

pH of the medium is a crucial factor for the activity of the polymerase enzyme responsible of PHB production (Bhagowati *et al*., 2015). It is clear that pH 7 is more favorable for PHB production where optimum PHB accumulation (75.66 ug/mL) and optimum cells growth (274 ug/mL) were obtained (Figure 5). Many authors have reported that pH 7 is the best pH for PHB accumulation using different bacterial strains (Mostafa *et al*., 2020; Singh *et al*., 2013). PHB synthesis decreased at acidic and alkaline pH.

The incubation temperature had a significant effect on PHB synthesis; this is due to the polymerase enzyme involved in the PHB polymerization, highly affected by the temperature changes—mainly the high temperatures (Getachew and Woldesenbet, 2016). The maximum PHB yield with a maximum cell growth and PHB accumulation were achieved at 37°C (Figure 6). Aly *et al.* (2013) reported the same results, using the bacterial strain *Bacillus cereus*. Singh *et al.* (2013), however, recorded that maximum PHB production by *Bacillus subtilis* was obtained at 40°C.

The nitrogen source affects PHB accumulation and bacterial cells growth. Bacteria accumulate PHB under stress conditions of nitrogen (Hungund *et al*., 2013). In our study, organic sources mainly tryptophane enhanced PHB accumulation by *Bacillus paramycoides*and cells growth (63.31 ug/mL and 226.33 ug/mL, respectively) in comparison with inorganic sources $(NH_4)_2SO_4$ (Figure 7). This may be explained by the fact that organic sources are considered as precursors of amino acids and bacterial growth factors (Patel *et al*., 2017). Ammonium sulfate was reported by Kritika *et al.* (2016) and Singh *et al.* (2013) to be the best nitrogen source for PHB accumulation

High contents of PHB (89.35 ug/mL) and cells biomass (260.33 ug/mL) were achieved using acid hydrolyzed date syrup in comparison with centrifuged and crude syrup (Figure 8). This is due to the low levels of simple sugars in non treated syrup in comparison with acid-treated syrup (McAdam *et al*., 2020). Sucrose hydrolysis increased with the increase in sulfuric acid concentration (Sen *et al*., 2019). The total rate of reducing sugars increased from 31.86 g/L to 78.86 g/L, 72.96 g/L, and 72.2 g/L after treatment of date syrup with $5MH_2SO_4$, 3MHCL, and 1.5 M H_2SO_4, respectively (Table 1). The same results were reported by Gomaa (2014) where acid preatreated molasses enhanced PHB accumulation in comparison with untreated molasses.

The carbon source is the major factor affecting PHB production costs (Aljuraifani *et al*., 2019; Gomaa, 2014). PHB production and cells growth are directly proportional to the syrup concentration (Figure 9). They reached their maximums at 8% (104.3 ug/mL and 319.73 ug/mL, respectively). This is due to the high content of sugar in concentrated date syrup. The use of the renewable resources depends on the nature of the complex material and the hydrolytic capacity of the bacterium enzymes (Belal, 2013). Gabr (2018) revealed that the best levels of PHB were accumulated by *Bacillus* sp. at 8% of date syrup. Therefore, the selection of an economically cost-effective carbon source is the key determining the final product market costs (McAdam *et al*., 2020). Date syrup by-products are abundant and inexpensive sources for PHB production that may decrease the costs of bioplastic production.

PHB is traditionally identified using HPLC on Aminex HPX-87H technique (Karr *et al*., 1983). The technique is based on the conversion of PHB into crotonic acid by concentrated sulfiric acid. Crotonic acid is measured in samples containing from 0.01 to 14 ug of PHB. Crotonic acid and pure PHB were used as standards. Chromatograms with the same peaks at the retention time 22.5 min were obtained (Figure 10), with crotonic acid, pure PHB, and produced PHB using *Bacillus paramycoides* and date syrup as carbon source which

confirms the conversion of date syrup into PHB by *Bacillus paramycoides*.

Conclusion

Date syrup, which is an inexpensive by-product, is a promising source for the production of PHB biopolymer. The latest is successefully produced using a bacterial strain isolated from soil, *Bacillus paramycoides*, and date syrup as carbon source. The best contents of PHB were reached after 96 h of incubation at 37°C, an agitation rate of 300 rpm, and pH 7 using acid preatreted date syrup at a concentration of 8%. Furthemore, more investigations are required to characterize the PHB chemical structure to determine the possibility of producing PHB at industrial levels from date syrup.

Acknowledgement

The authors are grateful to Mr Nasser ZAYDI, the chief of the hygiene laboratory of the state of Skikda, the engineers of the laboratory of animals biotechnologies, National Center for Biotechnology, Constantine, Algeria, Pr. Luis Isidoro Romero GARCIA, Dr. Kaoutar ABOUDI, and miss Hind ZINE EL ABIDINE from the faculty of Sciences, the university of Cadiz , Spain.

Authors contribution

All the authors contributed in the conception and design of the study. Bacterial isolation and PHB optimization conditions were performed by Leila Djerrab and Zohra CHEKROUD. Amer Rouabhia conducted HPLC analysis. Mohamed Abdesselem DEMS realized the statistical analysis. Mustapha Adnane SMADI provided necessary material and helped in realizing spectrophotometric analysis. Imane Attailia helped in the manuscript redaction. Faiçal Djazy provided some chemical products. The first draft of the manuscript was written by Zohra CHEKROUD and Leila DJERRAB. All the authors read and approved the final manuscript.

Conflict of interest

There is no conflict of interest to declare.

References

Abbès, F., Bouaziz, M.A., Blecker, C., Masmoudi, M., Attia,H. and Besbes, S., 2011. Date syrup effect of hydrolytic enzymes (pectinase/cellulase) on physico-chemical characteristics, sensory and functional properties. Lebensmittel-Wissenschaft & Technologie 44(8):1827–1834. https://doi.10.1016/j.lwt.2011.03.020

Adwitiya, P., Ashwini, P., Avinash, A.K., Badri, R., Kajal, D., Vomsi, P. and Srividya, S., 2009. Mutagenesis of *Bacillus thuringiensis* IAM 12077 to increase the production of poly backslash beta-hydroxybutyrate (PHB). Turkish Journal of Biology 33(3): 225–223. https://doi.org/10.3906/biy-0808-10

Aljuraifani, A.A., Berekaa, M.M. and Ghazwani, A.A., 2019. Bacterial biopolymer (polyhydroxyalkanoate) production from low-cost sustainable sources. Microbiology Open 8(6):e00755. https://doi.org/10.1002/mbo3.755

Ahmad, A., Naqvi, A., Jaskani, M.J.,Waseem, M.,Ali, E.,Khan, I.A., Manzoor, M.F., Siddeeg, A. and Aadil, R.M., 2021. Efficient utilization of date palm waste for the bioethanol production through *Saccharomyces cerevisiae* strain. Food Sciences & Nutrition 9(4):2066–2074. https://doi.org/10.1002/fsn3.2175

Aly, M.M., Albureikan, M., El Rabey, H. and Kabli, S.A., 2013. Effects of culture conditions on growth and poly –beta-hydroxybutyric acid production by *Bacillus cereus* MM7 isolated from soil samples from Saudi Arabia. Life Sciences Journal 10(4):1884–1891.

Anderson, A.J. and Dawes, E.A., 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiology Review 54(4):450–472. https://doi.org/10.1128/mr.54.4.450-472.1990

Ashraf, S., Ali, S. and Ul-Haq, I., 2015. Acidic pre-treatment of sugarcane molasses for citric acid production by *Aspergillus niger* NG-4. International Journal of Current Microbiology and Applied Sciences 4(6):584–595.

Baiano, A., 2014. Recovery of biomolecules from food wastes: a review. Molecules 19(9):14821–14842. https://doi.org/10.3390/molecules190914821

Bandaiphet, C. and Prasertsan, P., 2006. Effect of aeration and agitation rates and scale-up on oxygen transfer coefficient, kLa in exopolysaccharide production from *Enterobacter cloacae* WD7. Carbohydrate Polymers 66(2):216–228. https://doi.org/10.1016/j.carbpol.2006.03.004

Belal, E.B., 2013. Production ofpoly- β -hydroxybutyric acid (PHB) by *Rhizobium elti* and *Pseudomonas stutzeri*. Current Research Journal of Biological Sciences 5(6):273–284. https://doi.org/10.19026/crjbs.5.5429

Bhagowati, P., Pradhan, S., Dash, H.R. and Das, S., 2015. Production, optimization and characterization of polyhydroxybutyrate, a biodegradable plastic by *Bacillus* spp. Biosciences Biotechnology and Biochemistry 79(9):1454–1463. https://doi.org/10.1080/09168451.2015.1034651

Blunt, W., Levin, D.B. and Cicek, N., 2018. Bioreactor operating strategies for improved polyhydroxyalkanoate (PHA) productivity. Polymers 10(11):1197. https://doi.org/10.3390/polym10111197

Bouguedoura, N., Bennaceur, M., Babahani, S. and Benziouche, S.E., 2015. Date palm status and perspective in Algeria. In: Elkhayri, J.A., Jain, S.M. and Johnson, D.V. (eds.) Date palm genetic resources and utilization,volume 1 Africa and the Americas. Springer Dordrecht, pp. 125–168.

Chandrasekaran, M. and Bahkali, A.H., 2013. Valorization of date palm (Phoenix dactylifera) fruit processing by-products and

wastes using bioprocess technology–review. Saudi Journal of Biological Sciences 20(2):105–120. https://doi.org/10.1016/j.sjbs.2012.12.004

Chniti, S., Jemni, M., Bentahar, I., Shariati, M., Djelal, H., Amrane, A. and Hassouna, M., 2017. By-products of dates: optimization of the extraction of juice using response surface methodology and ethanol production. Journal of Microbiology Biotechnology and Food Sciences 7(2):204–208. https://doi.org/10.15414/jmbfs.2017.7.2.204-208

El-Nagga, E.A. and Abd El-tawab, Y.A., 2012. Compositional characteristics of date syrup extracted by different methods in some fermented dairy products. Annals of Agricultural Sciences 57(1):29–36. https://doi.org/10.1016/j.aoas.2012.03.007

Elsayed, N.S., Aboulwafa, M., Aboshanab,K. and Hassouna, N., 2013. PHB production in *Azomonas*, *Acinteobacter* and *Bacillus* species: isolation, screening and identification. Archives of Clinical Microbiology 4(5):5025–5035. https://doi.org/10.3823/271

Gabr, G.A., 2018. Isolation and identification of bacterial strains able to biopolymer polyhydroxybutyrate (PHB) production from soil of Al-Kharj probes, Saudi Arabia. Journal of Pharmaceutical Research International 21(6):1–11. https://doi.org/10.9734/jpri/2018/39532

Getachew, A. and Woldesenbet, F., 2016. Production of biodegradable plastic by polyhydroxybutyrate (PHB) accumulating bacteria using low cost agricultural waste material. BMC Research Notes 9:509. https://doi.org/10.1186/s13104-016-2321-y

Gomaa, E.Z., 2014. Production of polyhydroxyalkanoates (PHAs) by *Bacillus subtilis* and *Escherichia coli*grown on cane molasses fortified with ethanol. Brazilian Archives of Biology and Technology 57(1):145–154. https://doi.org/10.1590/S1516-89132014000100020

Gurieff, N. and Lant, P., 2007. Comparative life cycle analysis and financial analysis of polyhydroxyalkanoate production in mixed culture. Bioresource Technology 98(17): 3393–3403. https://doi.org/10.1016/j.biortech.2006.10.046

Gusakov, A.V., Kondratyeva, E.G. and Sinitsyn, A.P., 2011. Comparison of two methods for the determination of reducing sugars in the determination of carbohydrase activities. Journal of Analytical Chemistry 2011:283658. https://doi.org/10.1155/2011/283658

Hungund, B., Shyama, V.S., Patwardhan P. andSaleh, A.M., 2013. Production of polyhydroxyalkanoate from *Paenibacillus durus* BV-1 isolated from oil mill soil. Journal of Microbial and Biochemical Technology 5(1):13–17. https://doi.org/10.4172/1948-5948.1000092

Karr, D.B., Waters, J.K. andEmerich D.W., 1983. Analysis of poly-β-hydroxybutyrate in Rhizobium japonicum bacteroids by ion-exclusion high-pressure liquid chromatography and UV detection. Applied Environmental Microbiology 46(6): 1339–1344. https://doi.org/10.1128/aem.46.6.1339-1344.1983

Keshavarz, T. and Roy, I., 2010. Polyhydroxyalkanoates bioplastics with a green agenda. Current Opinion in Microbiology 13(3):321–326. https://doi.org/10.1016/j.mib.2010.02.006

Kritika, S., Pragya, R.,Nandini, P. and Priti, S., 2016. Optimization of PHB (poly-hydroxybutyrate) synthesis by Serratia sp. isolated from soil. International Journal of Current Microbiology and Applied Sciences 5(6):665–673. https://doi.org/10.20546/ijcmas.2016.506.072

Kundu, S., Panda, T., Majumdar, S.K., Guh, B. and Bandyopadhyay, K.K., 1984. Pretreatment of Indian cane molasses for increased production of citric acid. Biotechnology and Bioengineering 26(9):1114–1121. https://doi.org/10.1002/bit.260260915

Majzoobi, M., Karambakhsh, G., Golmakani, M.T., Mesbahi, G. and Farahnaky, A., 2020. Effects of level and particle size of date fruit press cake on batter rheological properties and physical and nutritional properties of cake. Journal of Agricultural Science and Technology 22(1):121–133. Available at: http://jast.modares.ac.ir/article-23-25558-en.html

Mantzouridou, F., Roukas,T. and Kekidou, P., 2002. Effect of the aeration rate and agitation speed on β-carotene production and morphology of Blakeslea trispora in a stirred tank reactor: mathematical modeling. Biochemical Engineering Journal 10(2): 123–135. https://doi.org/10.1016/s1369-703x(01)00166-8

McAdam, B., Fournet, M.B., McDonald, P. and Mojicevic, M., 2020. Production of polyhydroxybutyrate (PHB) and factors impacting its chemical and mechanical characteristics. Polymers 12(12): 1–20. https://doi.org/10.3390/polym12122908

Mohapatra, S., Maity, S., Dash, H.R., Das, S., Pattnaik, S., Rath C.C. and Samantaray, D., 2017. *Bacillus* and biopolymer: prospects and challenges. Biochemistry and Biophysics Reports 12: 206–213. https://doi.org/10.1016/j.bbrep.2017.10.001

Mohapatra, S., Pattnaik, S., Maity, S., Sharma, S., Akhtar, J., Pati, S., Samantaray, D.P. and Varma, A., 2020. Comparative analysis of PHAs production by *Bacillus megaterium* OUAT 016 under submerged and solid-state fermentation. Saudi Journal of Biological Sciences 27(5):1242–1250. https://doi.org/10.1016/j.sjbs.2020.02.001

Mohd Zahari, M.A.K., Ariffin, H., Mokhtar, M., Salihon, J., Shirai, Y. and Hassan, M.A., 2012. Factors affecting poly (3-hydroxybutyrate) production from oil palm frond juice by cupriavidus necator (CCUG 5 2 2 3 8 T). Journal of Biomedicine and Biotechnology 2012:125865. https://doi.org/10.1155/2012/125865

Mostafa, Y.S., Alrumman, S.A., Alamri, S.A., Otaif, K.A., Mostafa, M.S. and Alfaify A.M., 2020. Bioplastic (poly-3-hydroxybutyrate) production by the marine bacterium *Pseudodonghicola xiamenensis* through date syrup valorization and structural assessment of the biopolymer. Scientific Reports 10(1):8815. https://doi.org/10.1038/s41598-020-65858-5

Naheed, N., Jamil, N., Hasnain, S. and Abbas, G., 2012. Biosynthesis of polyhydroxybutyrate in *Enterobacter* sp. SEL2 and the bacterium Enterobacteriaceae sp. PFW1 using sugar cane molasses as a carrier. African Journal of Biotechnology 11(16):3321–3332. https://doi.org/10.5897/AJB11.1405

Nancib, A., Nancib, N., Boubendir, A. and Boudrant, J., 2015. The use of date waste for lactic acid production by a fed-batch culture using *Lactobacillus casei* subsp. rhamnosus. Brazilian Journal of Microbiology 46(3):893–902. https://doi.org/10.1590/S1517-838246320131067

Narayanan, M., Kumarasamy, S., Ranganathan, M., Kandasamy, S., Kandasamy, G. and Gnanavel, K., 2020. Enzyme and metabolites attained in degradation of chemical pesticides ß- Cypermethrin

by *Bacillus cereus*. Materials Today: Proceedings 33(7): 3640–3645. https://doi.org/10.1016/j.matpr.2020.05.722

Omar, S., Rayes, A., Eqaab, A., Vob, I. and Seinbüchel, A., 2001. Optimization of cell growth and poly (3-hydroxybutyrate) accumulation on date syrup by a Bacillus megaterium strain. Biotechnology Letters 23(14):1119–1123. https://doi.org/10.1023/a:1010559800535

Patel, N., Patel, P. and Desai, R., 2017. Detection and characterization of PHB (polyhydroxybutyrate) producers halophilic bacteria isolated from marine water sample of Valsad District. International Journal of Pharma and Bio Sciences 8(3): 1100–1108. https://doi.org/10.22376/ijpbs.2017.8.3.b1100-1108

Sen, K.Y., Hussin, M.H. and Baidurah, S., 2019. Biosynthesis of poly (3-hydroxybutyrate) (PHB) by *Cupriavidus necator* from various molasses pretreated as a carbon source. Biocatalysis and Agricultural Biotechnology 17:51–59. https://doi.org/10.1016/j.bcab.2018.11.006

Sharma, L., Singh, A.K., Panda, B. and Mallick, N., 2007. Process optimization for poly β-hydroxybutyrate production in a nitrogen fixing cyanobacterium, Nostoc muscorum using response surface methodology. Bioresource Technology 98(5):987–993. https://doi.org/10.1016/j.biortech.2006.04.016

Singh, G., Kumari, A., Mittal, A., Yadav, A. and Aggarwal, N.K., 2013. Poly β-hydroxybutyrate production by *Bacillus subtil*is NG220 using sugar industry waste water. BioMedResearch International 2013: 952641. https://doi.org/10.1155/2013/952641

Tallon, P., Magajna, B., Lofranco,C. and Leung, K.T., 2005. Microbial indicators of faecal contamination in water: a current perspective. Water, Air and Soil Pollution 166:139–166. https://doi.org/10.1007/s11270-005-7905-4

Thapa, C., Shakya, P., Shrestha, R., Pal, S. and Manandhar, P., 2018. Isolation of polyhydroxybutyrate (PHB) producing bacteria, optimization of culture conditions for PHB production, extraction and characterization of PHB. Nepal Journal of Biotechnology 6(1):62–68. https://doi.org/10.3126/njb.v6i1.22339

Vu, D.H., Wainaina, S., Taherzadeh, M.J., Åkesson, D. and Ferreira, J.A., 2021. Production of polyhydroxyalkanoates (PHAs) by *Bacillus megaterium* using food waste acidogenic fermentation-derived volatile fatty acids. Bioengineered 12(1):2480–2498. https://doi.org/10.1080/21655979.2021.1935524

Werlang, E.B., Moraes, L.B., Muller, M.V.G., Julich, J., Corbellini, V.A., Neves, F.D.F., de Souza, D., Benitez, L.B. and Schneider, R.D.C.D., 2021. Polyhydroxybutyrate (PHB) production via bioconversion using *Bacillus pumilus* in liquid phase cultivation of the biomass of *Arthrospira platensis* hydrolysate as a carbon source. Waste and Biomass Valorization 12:3245–3255. https://doi.org/10.1007/s12649-020-01213-z