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Strategies to Enhance Polyhydroxyalkanoate Production from Sugarcane Molasses by *Cupriavidus necator* 11599

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Abstract

The production of polyhydroxyalkanoate (PHA) is an opportunity to gradually replace some plastics produced from fossil resources. The use of agro-industrial waste to produce PHA is one of the most efficient techniques, because the cost of production is high. Molasse is waste from the sugar industry. It has already been transformed into PHA via fermentation. But *Cupriavidus necator is* unable to produce PHA from raw molasse. So, several authors have tried to overcome this problem by pretreating molasse before production. In this study, fermentation was conducted in a shake-flask with *Cupriavidus necator.* Three types of pretreatments of molasse were conducted to enhance PHA production: i) sulfuric acid pretreatment; ii) enzymatic pretreatment and iii) pretreatment with activated carbon. Molasse pretreated accumulates up to a maximum PHA content of 64.56, 75.64 and 58.14 wt.% respectively with (15:100) ratio for acid, (15:100) ratio for enzyme and (20:100) ratio for activated carbon. The obtained result showed an enhancement of PHA production from sugarcane molasses.

Keywords

Molasse, Pretreatment, Fermentation, PHA, *Cupriavidus necator*

1. Introduction

Current packaging is made from petrochemical plastics, which are toxic to the

environment [1]. Since the full-scale production of petroleum based plastics, they have been the main culprits of marine pollution [2]. Their daily discharge into nature causes ecotoxicity, not only due to plastic waste but also due to the degradation products of plastic, namely micro and nanoplastics. The aquatic accumulation of nanoplastics poses a threat to the health of exposed invertebrates (such as mussels, algae, and oysters). They consume them as food, and more species (such as seals and turtles) [3] get trapped in plastic. In view of the above-mentioned problems, a potential solution would be to replace petroleum plastics with biodegradable plastics [4].

Bioplastics are natural biopolymers synthesized and catabolized by various microorganisms [5]. Polyhydroxyalkanoates (PHAs) are the most popular biopolymers. They are thermoplastic and possess the same properties as those appreciated in petrochemical plastics. Over the last few decades, they have gained substantial interest due to their biocompatibility and biodegradability. So they can extend their industrial applications to the medical fields [6] and food packaging [7]. Unfortunately, the production of PHAs is expensive [8], with the cost being 4 to 9 times higher than the price of polyethylene [9]. The total production cost relies on the cost of the carbon source, which accounts for about 50% - 60% [10]. Thus, a new approach involves using renewable carbon resources derived from agriculture and/or industrial waste as a carbon source for PHA production [11]. This approach has the advantage of making the production of PHA more economical and reducing the volume of waste by giving it added value [12]. In this sense, many wastes have been used for producing PHA. Like cashew apple juice [13], cassava peel starch [14], glycerin [15], orange peel [16], and molasses [17].

Molasses were experimented with, but no conclusive result was found due to the complexity of this substrate. *Cupriavidus necator* is unable to assimilate sucrose, a main component of molasses [18]. This problem can be solved by mutagenesis, on the one hand. Genes bacteria are modified. On the other hand, this problem can be solved by pretreating molasses before fermentation [19]. This study has been focused on pretreatment of molasses. Hence, various pretreatments of molasses are performed at different rates.

The main objective is to show how each pretreatment enhances PHA production and which of them is efficient and suitable for *Cupriavidus necator* NCIMB 11599.

2. Materials and Methods

2.1. Bacterial Strain and Growth Condition

Cupriavidus necator NCIMB 11599 was used for PHA production via fermentation. The strain was cultured by streaking on medium having the following composition (g/L): 6 $Na_2HPO_4{\cdot}7H_2O$, 2.4 KH_2PO_4, 1 NH_4Cl, 0.5 $MgSO_4{\cdot}7H_2O$, 0.01 $FeCl_3{\cdot}6H_2O$ and 0.01 $CaCl_2$. The mineral medium was supplemented with 20 g/L agar and 20 g/L glucose. The $FeCl_3.6H_2O$ and $CaCl_2$ solution was sterilized with a 2 µm polyethersulfone filter. Glucose, NH_4Cl and $MgSO_4{\cdot}7H_2O$ autoclaved separately at 121˚C for 15 min. These solutions were mixed aseptically after cooling.

The pH of the medium was maintained at 6.8 with NaOH 4N and H_2SO_4 4N. Plate inoculated with strain was incubated at 30˚C ± 1˚C for 3 days and then stored at 4˚C for further study.

2.2. Sugarcane Molasses Preparation

The raw sugarcane molasses used in this work came from the "SUCRIVOIRE" industry in Zuénoula (Côte d'Ivoire). The molasses was diluted in 1:4 (v/v) ratio to obtain 80 g/L of initial reducing sugar before all utilization in this study. This dilution process allows easy handling during pretreatment and fermentation. Sugarcane molasse diluted was stored at 4˚C for further use.

2.3. Sugarcane Molasses Pretreatment

Sugarcane molasse diluted previously was pretreated with acid (sulfuric acid H_2SO_4), with enzyme (baker's yeast: Fleischmann—AB Foods) and with activated carbon (OLC 12X40 (OLC)).

2.3.1. Pretreatment with Acid (Sulfuric Acid H_2SO_4)

Experiments for acid pretreatment (H_2SO_4) were conducted in a 500 mL flask containing, 100 mL of molasses diluted that was mixed well. Sugarcane molasse diluted was acidified with various volume 5, 10, 15 mL of 1.5 N H_2SO_4 [17] and the mix was adjusted to pH of approximately 6.8 with NaOH 4N. The mixture was incubated in a water bath for 1 h (90˚C, 150 rpm) and then cooled to 30˚C. Subsequently, the separation into solid and liquid phases was achieved by centrifugation at 3500 rpm for 15 min. The recovered supernatant was put into the oven for 1 h (105˚C).

2.3.2. Pretreatment with Enzyme

Enzymatic pretreatment (EZ) with Saccharomyces cerevisiae baker's yeast (Fleischmann—AB Foods) consisted of an invertase-rich dry yeast preparation according to Dalsasso *et al.* [20]. Initially, the dry yeast was hydrated to a ratio of 1:3 (w/v), maintaining the suspension in a water bath for 2 h (60˚C, 150 rpm) to promote autolysis. Then 3, 10, 15 mL of yeast suspension was added to 100 mL of solution of sugarcane molasses in a 500 mL flask containing. Then, incubated in a water bath for 5 h (60˚C, 150 rpm) followed by cooling to room temperature (30˚C) for 30 min. The solids were separated by centrifugation at 8000 rpm for 30 min. The recovered supernatant was put in the oven for 5 min (85˚C) to ensure the inactivation of the enzyme and residual yeast.

2.3.3. Pretreatment with Activated Carbon OLC 12X40 (OLC)

The pretreatment with activated carbon (AC) is based on the method of Farmani *et al.* [21] with modification of temperature, time and pretreatment stirring. So, the pretreatment's temperature was 90˚C instead of 75˚C, pretreatment's time was 1h instead 15 min and pretreatment's stirring were 140 rpm instead 120 rpm. The pretreatment was done, with commercial reference carbon OLC 12X40 (OLC). AC comes from Calgon Carbon Corporation, a USA manufacturer. It was added

to 100 mL of sugarcane molasses, at the concentration of 1.5, 13, 20 % (w/v). Then, the mixture was incubated in a water bath for 1 h (90˚C, 140 rpm). Then the mixture was filtrated through filter paper (1.6 µm pore diameter) placed on the flat bottom of the Büchner funnel connected to a vacuum pump (Heidolph). The obtained filtrated solution was cooled to room temperature and the pH was adjusted to 6.8 with NaOH (4N). Then a volume was taken (about 10 mL) to determine the physicochemical characterization such as glucose, fructose, sucrose, and some minerals. To prevent microbial activity, all obtained filtrates were subjected to sterilization to 121˚C for 15 min.

2.4. Rotary Shaker Experiments

Fermentation methodology was conducted in rotary shaker, for PHA biosynthesis from molasses. All broth fermentation were conducted in the incubator shaker model (INFORS HT multitron pro) set at 200 rpm at 30˚C. The bacterial strain was initially grown in two precultures before PHA production.

2.4.1. Preculture 1

A loop full of *Cupriavidus necator* NCIMB 11599 from the plate was used as inoculum for pre-culture 1 contained in the Erlenmeyer flasks and then incubated. The composition of mineral medium (g/L) for preculture 1 was: 6 $Na_2HPO_4{\cdot}7H_2O$, 2.4 KH_2PO_4, 1 NH_4Cl, 0.5 $MgSO_4.7H_2O$, 0.01 $FeCl_3{\cdot}6H_2O$, 0.01 $CaCl_2$. Mineral medium was supplemented with 20 g/L glucose. The $FeCl_3{\cdot}6H_2O$ and $CaCl_2$ solution was sterilized with a 2 µm polyethersulfone filter. Glucose, NH_4Cl and $MgSO_4{\cdot}7H_2O$ autoclaved separately at 121˚C for 15 min. These solutions were mixed aseptically after cooling. The pH of the medium was maintained at 6.8 with NaOH 4N et H_2SO_4 4N. The Erlenmeyer flasks were incubated for 24 h.

2.4.2. Preculture 2

Then actively growing cells from preculture 1 (10 % v/v) were used as inoculum for preculture 2 [22]. The composition of the mineral medium for preculture 2 was the same as preculture 1 except molasses (molasses pretreated or not) were used in place of glucose (15, 20, 25 g/L) as carbon source. The incubation conditions were the same as those for preculture 1 [23].

2.4.3. Production of PHA

Preculture 2 (10 % v/v) was used as an inoculum for 300 mL of production medium contained in 2L Erlenmeyer flask. For the PHA production, molasses as well as pretreated molasses were used at a concentration of 15, 20, 25 g/L were used as carbon source. In the case of pretreated molasses three (3) types *i.e.* pretreated with acid (sulfuric acid (**H_2SO_4**)), enzyme (baker's yeast (**EZ**)) and activated carbon OLC 12X40 (OLC): **AC** were used. The composition of the mineral medium composition and incubation conditions were the same as in preculture 1 and 2. The pH of the medium was maintained at 6.8 NaOH 4N et H_2SO_4 4N. The Erlenmeyer flasks were incubated for 24 h. Fermented broths samples 20 mL, were drawn each 24 h to measure the biomass concentration, the reducing sugar con-

centration and the PHA concentration.

2.5. Analytical Methods

2.5.1. Determination of Cell Dry Weight

Biomass concentration, expressed as cell dry weight (CDW), was measured according to the standard method [24]. At different time (t), 20 mL of each sample withdrawn from fermented broth was centrifuged at 7000 rpm for 15 min. After centrifugation, two parts were formed: liquid as the supernatant and pellet at the bottom. Each pellet was suspended in distilled water mixed well and centrifuged at 7000 rpm for 15 min. The washed pellet was collected and placed in a previously weighed empty crucible, then dried at 60˚C in oven for 24 h and to obtain the dry weight. CDW (g/L) was calculated in Equation (1).

$$\text{CDW} = \frac{m_1 - m_0}{V_b} \tag{1}$$

m_0: mass (g) of empty crucible,

m_1: mass (g) of crucible with dried pellet,

V_b: fermented broth volume (L).

2.5.2. Determination of PHA

PHA concentration was measured using the standard methanol and chloroform extraction method described by Comeau *et al.* [25]. Mass of each different dried pellet obtained previously and PHA standards (Aldrich from Sigma-Aldrich International GmbH) were put in individual test tubes. For each mass, 2 mL acidified methanol followed by 1 mL of chloroform was added. The mixture was heated in an oil bath at 95˚C for 2 h. Then 1 mL of chloroform and 1 mL of water were added. The samples were well mixed on a Vortex mixer and left on the bench for phase separation. The PHA dissolved in chloroform phase was recovered from the bottom of the tube. The concentration of PHA (g/L) was then determined by gas chromatography analysis. GC made by Agilent Technologies, model 7890B with flame ionization detector (FID).

2.5.3. Determination of Reducing Sugar

Reducing sugar (RS) concentration in the broth during fermentation was analyzed by using the DNS (3,5 dinitrosalicylic acid) method [26]. The supernatant of each sample of the fermented broth obtained during the determination of cell dry weight, was diluted according to the concentration of reducing sugars. To one mL of each diluted sample, 3 mL of DNS reagent was added. The mixture was placed in a water bath at 95˚C for 5 min. After cooling, the concentration of reducing sugar was determined by using a spectrophotometer at a wavelength of 540 nm with glucose as standard.

3. Results and Discussion

3.1. Chemical Characterization of Molasses

Molasses is rich in glucose, fructose and sucrose (**Table 1**). Glucose and fructose

are easily assimilated by bacteria, because they are simple sugars. But sucrose is a complex sugar, composed of glucose and fructose. The *Cupriavidus necator* is unable to use it directly. This inability will be overcome by pre-treating the molasses before fermentation in order to release the bound glucose and fructose [27]. Molasses is also composed of minerals. Those in deficit will be able to be supplemented [13]. The pH of molasses is 5.6. As this value is lower than 6.8 (growth pH of the *Cupriavidus necator*), it is not suitable for bacterial growth [28]. However, it can be regulated with sodium hydroxide.

Table 1. Chemical characterization of sugarcane molasses.

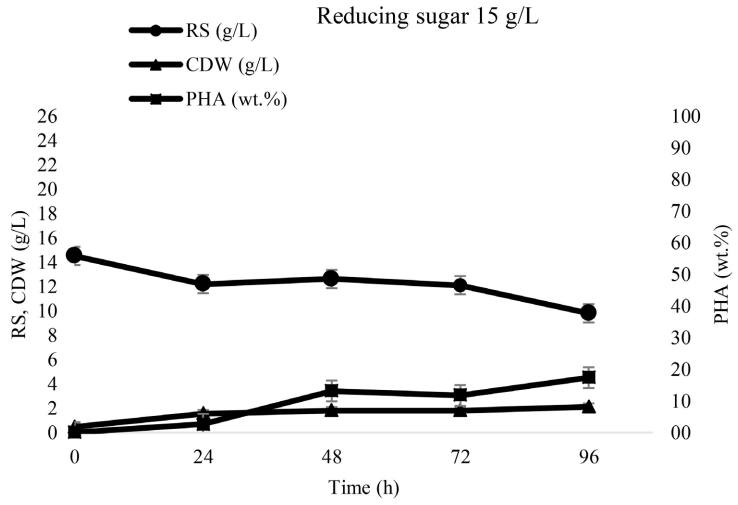
Composition	Concentration	Composition	Concentration
TC (g/L)	508.1	**Calcium (mg/L)**	9450
TOC (g/L)	508	**Cobalt (mg/L)**	0.6
IC (g/L)	0.07	**Chromium (mg/L)**	0.72
NT (g/L)	8.16	**Copper (mg/L)**	0.81
NH_4 (g/L)	-	**Iron (mg/L)**	184
Glucose (g/L)	210	**Potassium (mg/L)**	61,200
Fructose (g/L)	140	**Magnesium (mg/L)**	3230
Lactose (g/L)	-	**Manganese (mg/L)**	38.84
Sucrose (g/L)	130	**Sodium (mg/L)**	140
Galactose (g/L)	-	**Phosphorus (mg/L)**	520
Xylose (g/L)	-	**Sulfur (mg/L)**	2310
Trehalose (g/L)	-	**Silicon (mg/L)**	139
Aluminium (mg/L)	7	**Strontium (mg/L)**	23.12
Boron (mg/L)	2.3	**Zinc (mg/L)**	10.1
Barium (mg/L)	6.9		

TC: Total Carbon TOC: Total Organic Carbon IC: inorganic Carbon TN: Total Nitrogen.

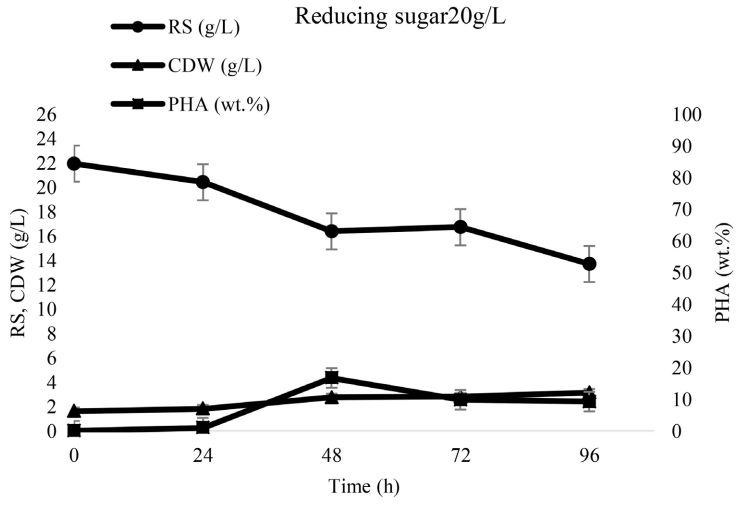
3.2. Effect of Reducing Sugar Concentration on PHA Production

The aim of this section is to find out if the concentration of reducing sugar can influence the accumulation of PHA. Also, to determine which reducing sugar concentration is suitable for obtaining an abundant quantity of PHA. The CDW concentration (g/L), the PHA percentages (wt.%) are considered as answers. **Figure 1** shows the results obtained.

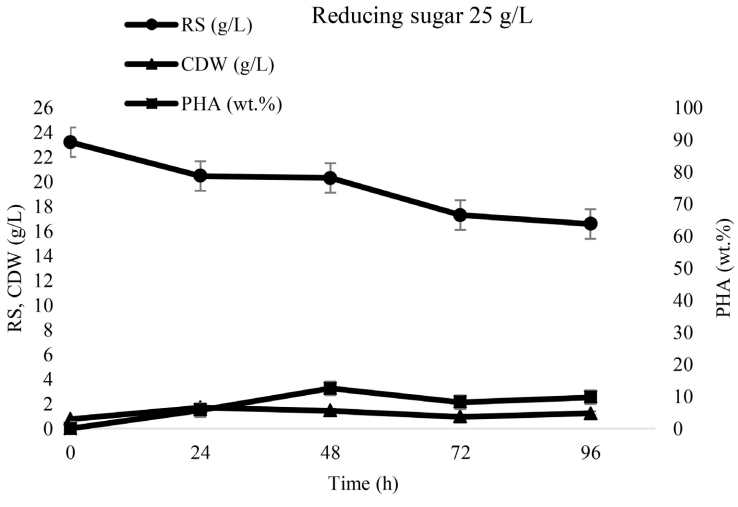
The highest PHA percentages are obtained with a reducing sugar concentration of 15 g/L with 17.33 wt.% at 96 h (**Figure 1(a)**). Others concentration 20 g/L and 25 g/L gave respectively only 16.64 wt.% and 12.52 wt.% at 48 h (**Figure 1(b)** and **Figure 1(c)**). Therefore, the initial reducing sugar (RS) concentration of 15 g/L was selected for PHA production for further work.



(a)



(b)



(c)

Figure 1. Effect of various reducing sugar concentration of molasses (a) 15 g/L, (b) 20 g/L and (c) 25 g/L on the PHA production.

3.3. Effect of Pretreatment of Molasses on Sugar Content and Mineral Content

Pretreatment of substrates before fermentation is a common method to help the bacteria to assimilate the substrate and to produce the biopolymer. For example, the pretreatment of agro-industrial by-products [29], the pretreatment of cassava peel [14] and also molasse [17]. **Table 2** shows the glucose, fructose, sucrose and mineral content of diluted molasse (untreated molasse) and molasse pretreated with sulfuric acid and enzyme. The sulfuric acid and the enzyme reduced the sucrose concentration from 32,500 mg/L to 31,000 and <400 mg/L respectively. This reduction shows the hydrolysis of sucrose. Sucrose compounds two molecules, linked by a bond, which break during hydrolysis. Hydrolysis gives glucose and fructose [30]. Therefore, the concentration of fructose rises from an initial concentration of 35,000 mg/L in diluted molasses to 62,000 mg/L in molasses pretreated with acid and 111,000 mg/L in molasse pretreated with an enzyme. However, the concentration of glucose decreased from 52,500 mg/L to 18,000 mg/L in the molasses pretreated with acid and 44,000 mg/L in the molasses pretreated with an enzyme. Because the kinetics of sucrose are not always linear [31]. While glucose and fructose are produced, the glucose can undergo an isomerization due to temperature and pressure, to give fructose [32]. Also, in the case of pretreatment with sulfuric acid, glucose can undergo Maillard reaction [33]. In the case of pretreatment with the enzyme, yeast which hydrolyze the sucrose can consume the glucose present [34] [35]. Also, the pretreatments carried out led to a reduction in the concentration of some minerals like Barium, Calcium, Chromium, Iron, Potassium, Magnesium, Manganese, Sulfure and Strontium

Table 2. Sugar and mineral content of molasses before and after pretreatment with enzyme and acid.

Composition	Molasses raw	Molasses diluted	Molasses EZ	Molasses H_2SO_4
Glucose (mg/L)	210,000	52,500	44,000	18,000
Fructose (mg/L)	140,000	35,000	115,000	62,000
Sucrose (mg/L)	130,000	32,500	<400	31,000
Barium (mg/L)	6.9	1.725	1.34	1.35
Calcium (mg/L)	9450	2362.5	1697	1707
Chromium (mg/L)	0.725	0.181	0.131	0.129
Iron (mg/L)	184	46	36.1	35.3
Potassium (mg/L)	61,200	15,300	12,360	12,110
Magnesium (mg/L)	3230	807.5	647	645
Manganese (mg/L)	38.835	9.709	7.40	7.40
Sulfure (mg/L)	2310	577.5	483	1388
Strontium (mg/L)	23.119	5.780	4.59	4.59

Molasses EZ: molasse pretreated with enzyme; Molasses H_2SO_4: molasse pretreated with sulfuric acid.

(Table 2). The acid and enzyme pretreatments were accompanied by heat treatment in the oven. Heat treatment can induce the formation of complexes between the minerals and other compounds present in the molasses. These complexes can have effects on the free minerals and can influence their availability (reduction of concentration). In some cases, heat treatment can lead to the degradation of minerals present in molasses by altering the structure of the minerals [36].

The composition of some minerals before and after pretreatment with activated carbon is in Table 3. With activated carbon, after pretreatment a reduction of minerals (Barium, Calcium, Chromium, Copper, Potassium, Magnesium, Manganese, Phosphorus, Sulfur, Strontium and Zinc) was observed. This reduction was also observed by another author [37], who pretreated molasses with activated carbon and used it as the sole carbon source for genetically modified *R. eutropha* NCIMB11599 and *R. eutropha* 437-540. Activated carbon is known for its ability to reduce heavy metals and pigments [38] [39]. Some authors have shown a reduction in the quantity of Calcium and potassium after treating molasses with activated carbon at different concentrations [21]. This is an commonly adsorbent used for the decolorization of juice, molasses and syrups in the sugar industry [40]. Through an adsorption mechanism, it can remove different types of impurities such as colorants, turbidity compounds, proteins, phenolic compounds and anthocyanins, melassigenic elements [41]. These compounds are basically hydrophobic and negatively charged, so they tend to bind to positively charged functional groups on the surface of Activated carbon [21].

Table 3. Mineral content of molasses before and after pretreatment with activated carbon.

Composition	Molasses raw	Molasses diluted	Molasses AC
Barium (mg/L)	6.9	1.725	1.57
Calcium (mg/L)	9450	2362.5	1885
Chromium (mg/L)	0.725	0.181	0.157
Copper (mg/L)	184	46	42.7
Potassium (mg/L)	61,200	15,300	14,110
Magnesium (mg/L)	3230	807.5	727
Manganese (mg/L)	38.835	9.709	7.8
Phosphorus (mg/L)	520	130	128
Sulfur (mg/L)	2310	577.5	526
Strontium (mg/L)	23.118	5.780	5.43
Zinc (mg/L)	10.1	2.525	1.68

Molasses AC: molasse pretreated with activated carbon.

3.4. Effect of Various (Acid/Molasses) Ratios on Bacterial Growth and PHA Production

The effect of various ratios of acid pretreatment on the production of PHA was studied (Figure 2). Molasse pretreated with acid at ratio (5:100), ratio (10:100)

and ratio (15:100) were utilized as a carbon source for bacterial fermentation. Untreated molasses (M) is chosen as the control due to its low capacity to produce PHA. The first ratio (5:100) gave 51.44 wt.% PHA. This percentage is higher than PHA (17.33 wt.%) obtained with molasse without pretreatment (M). Also, compared with Sen [17], which obtained 20 wt.% PHA with the same volume of acid for its molasses pre-treatment, this study has higher PHA production. This improvement in PHA is due to the hydrolysis of sucrose in the presence of a catalyst (H+) from sulfuric acid. As a strong acid, sulfuric acid releases H^+ ions (protons) into an aqueous solution. Its reaction is due to its ability to give and accept protons in chemical reactions. In the presence of water in molasses, it can cause the dehydration of the sugars present, forming simpler compounds and decomposition products. The mechanism of acid hydrolysis is described by the protonation of the glycosidic oxygen atom leading to the cleavage of the disaccharide [42]. Sulfuric acid acts as a catalyst in the reaction. The first step consists of a reaction between sulfuric acid and sucrose, forming sucrose acid. The saccharic acid formed is unstable and undergoes dehydration. The water molecules are eliminated from the positions where the sucrose was bound, causing the glycosidic bond between glucose and fructose to split. This leads to the formation of free glucose and free fructose. Hydrolysis breaks down the sucrose into D-glucose and D-fructose. As a result, pretreated molasses doesn't contain sucrose, but free glucose and fructose, which can be used directly by *Cupriavidus necator* NCIMB 11599.

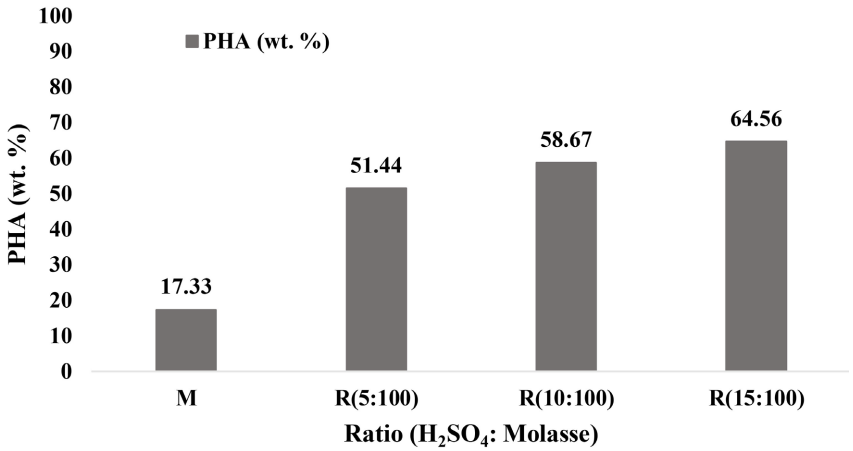


Figure 2. Effect of various (acid/molasses) ratios on PHA production.

The PHA percentage generally increased with increasing (acid/molasses) ratio (**Figure 2**). So, the increase in PHA production was positively correlated with the increase in the amount of sulfuric acid. The maximum PHA content of 64.56 wt.% was obtained with the (15:100) ratio. This is because with a low quantity of sulfuric acid, some sucrose is not hydrolyzed completely. There is a lack of simple sugar in the medium, limiting factors for the bacteria. The bacteria are unable to hydrolyze and use the residual sucrose [18] [43]. Therefore, the volume of acid influences the quantity of simple sugars in the medium. With the 15:100 ratio, the acid

was able to hydrolyze more sucrose. To produce simple sugars in the medium, which have been used by the bacteria. Sulfuric acid is often used to hydrolyse sucrose into glucose and fructose in the culture medium. These simple sugars can then be used as a carbon source by PHA-producing bacteria. The efficiency of this hydrolysis can directly influence the availability of carbon substrates for PHA production. The amount of sulfuric acid added for hydrolysis must be carefully controlled. Insufficient concentration can lead to incomplete hydrolysis of sucrose and limit the availability of carbon for PHA synthesis [44]. Clearly, the addition of sulfuric acid can decrease the pH value of the medium due to its acidic nature. It is essential to monitor and regulate the pH to ensure that it remains within the optimal range for growth and PHA production for specific bacteria. The concentration of sulfuric acid used for sucrose hydrolysis must be carefully chosen according to the amount of sucrose you wish to hydrolyze. The aim is to obtain a concentration of sulfuric acid that is sufficient to catalyze sucrose hydrolysis efficiently, while avoiding using excessive concentration that could be harmful.

3.5. Effect of Various (Enzyme/Molasses) Ratios on Bacterial Growth and PHA Production

The effect of (enzyme/molasses) ratios on PHA production is shown in **Figure 3**. The first ratio (3:100) gives 71.79 wt.% PHA, higher than 17.33 wt.% PHA with molasse unpretreated (M). Similarly, Dalsasso [20] observed a percentage of 75 wt.% PHA, in the same range as this study with the same volume of enzyme solution. Enzymatic pretreatment with baker's yeast (Fleischmann - AB Foods) increases PHA production through the action of the yeast Saccharomyces cerevisiae. It possesses enzymes (2-β-Dfructofuranosyl-1-α-D-glucopyranosidase) capable of hydrolyzing sucrose [45] [46]. When yeast is added to sucrose, the invertase enzymes begin to act on the sucrose molecules present [47]. The enzyme breaks the bond between glucose and fructose, releasing glucose and fructose [48], then accessible to *Cupriavidus necator*. The same reaction is observed in bread-making. The enzyme recognizes the sucrose molecule as a substrate. Because each enzyme is specific to a particular substrate [49].

The PHA percentage generally increased with increasing (enzyme/molasses) ratio (**Figure 3**). So, the increase in PHA production was positively correlated with the increase in the amount of sulfuric acid. The maximum PHA content of 75.09 and 75.64 wt.% was obtained respectively with the (10:100) and (15:100) ratio. This is because with a low quantity of enzyme, some sucrose is not hydrolyzed completely. The results show that after the 10:100 ratio, there isn't any significant increase in PHA production. Because the activity of the enzyme can be inhibited by a high concentration of substrate (molasses) at a low concentration of enzyme, known as "substrate inhibition". At a low concentration, the occupation of active sites on the enzyme-substrate complex is low and the rate of reaction is directly related to the number of sites occupied. As the enzyme-substrate complex forms, as the reaction rate increases, and the sugars are available for the bacteria. This is

the case with the (15:100) ratio. However, there is a limit to the speed of reaction when the substrate concentration is high. This is because all the enzyme's active sites are already occupied by substrate molecules [50].

Baker's yeast (Fleischmann - AB Foods) increases PHA production thanks to the action of Saccharomyces cerevisiae yeast, which possesses the enzyme invertase or 2-β-Dfructofuranosyl-1-α-D-glucopyranosidase, capable of hydrolyzing sucrose into glucose and fructose [45] [46].

The yeast invertase (enzyme) acts in several stages. Enzyme binds to the sucrose in the molasses. The sucrose is bound precisely to the invertase active site, via specific interactions such as hydrogen bonds and Van der Waals forces. The active site in turn contains amino acids, which enable it to consolidate the substrate and position the glycosidic bond for hydrolysis. Next, key catalytic residues from the enzyme protonate the oxygen atom of the glycosidic bond. This weakens the bond between glucose and fructose. Then a molecule of water enters the active site, and its oxygen attacks the glycosidic bond. In the end, the glycosidic bond is broken, releasing one molecule of glucose and one of fructose. It therefore improves the accessibility of glucose and fructose to the bacteria. It thus facilitates and activates its metabolism [48].

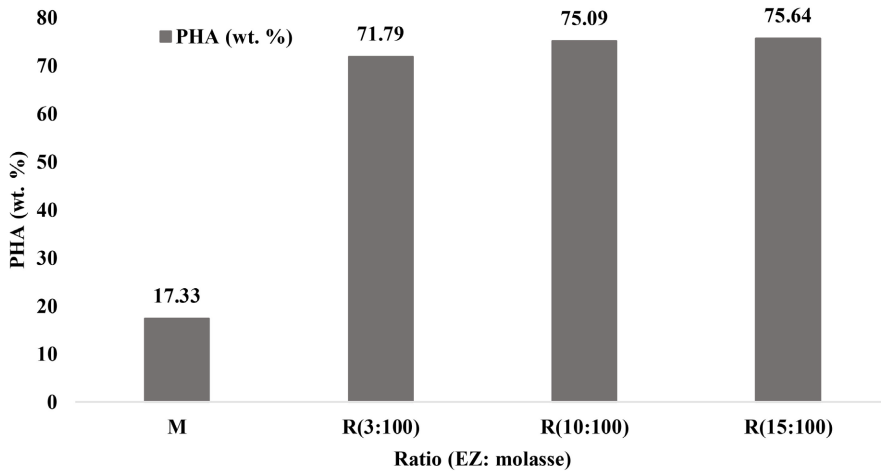


Figure 3. Effect of various (enzyme/molasses) ratios on PHA production.

3.6. Effect of Various (Activated Carbon/Molasses) Ratios on Bacterial Growth and PHA Production

The effect of (activated carbon/molasses) ratios on PHA production is shown in Figure 4. The first ratio (1.5:100) gives 7.46 wt.% PHA, lower than 17.33 wt.% PHA with molasse unpretreated (M). Of course Farmani [21] didn't pretreat molasses with activated carbon to produce PHA. But he used the same quantities of activated carbon to eliminate the impurities in the molasses. However, in this study, the first ratio (1.5:100) didn't remove impurities in the molasses. So, the bacteria didn't grow and didn't produce PHA. The impurities in molasses are calcium (Ca), potassium (K) ions… They are necessary for microbial growth, but in

high concentrations they are considered like impurities. Because they affect the growth of the host strain and the production of PHA [19] [27]. Residual pesticides, which can be found in molasses, from agricultural practices are also impurities. They can also affect the growth of bacteria and PHA production [51] [52]. High concentrations of heavy metals can also damage bacterial cells and disrupt their metabolism, which can hinder PHA production [53] [54].

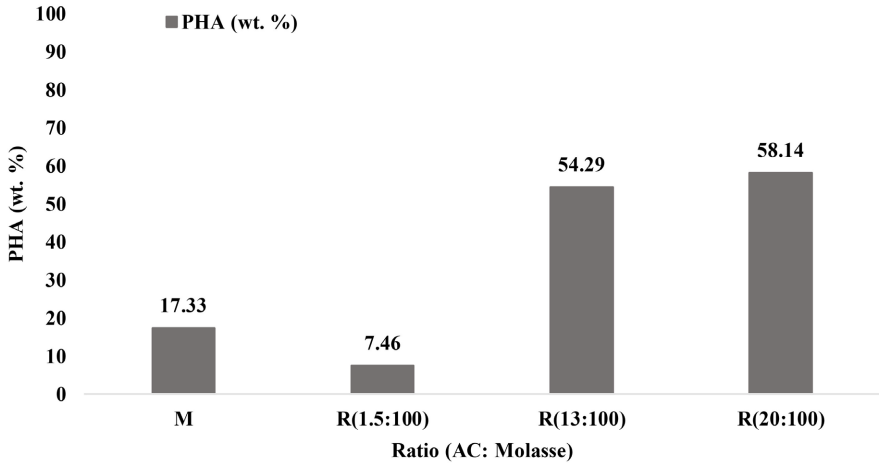


Figure 4. Effect of various (activated carbon/molasses) ratios on PHA production.

From the second ratio (13:100), the PHA percentage increased with increasing (activated carbon/molasses) ratio (**Figure 4**). The maximum PHA content of 58.14 wt.% was obtained with the (20:100) ratio. Increasing the quantity of activated carbon increases the number of active sites on its surface. The proportion of activated carbon used to pretreat solutions has a strong influence on the elimination of inhibiting compounds. This is due to the greater surface area available (when there is a high concentration of activated carbon) for adsorption of a large quantity of inhibiting compounds [55] [56]. PHA production is improved because the activated carbon removed impurities through a process called adsorption. Its adsorption properties enable it to trap and retain impurities on its surface. Activated carbon has a porous structure with a very large internal surface area. This porous structure creates a large specific surface area, where undesirable molecules can bind [57]. Impurities present in molasses have an affinity for the activated carbon surface due to their chemical properties. Attractive forces, such as van der Waals forces, dipole-dipole interactions and hydrogen bonds, also facilitate the attachment of these molecules to the activated carbon surface [58] [59].

Activated carbon has a direct influence on the metabolism of the bacteria and the enzymes involved in PHA production. This is because the inhibitors reduced by activated charcoal, in high concentrations, unbalanced the metabolism and inactivated the action of the enzymes. There are several ways of inactivating the enzyme. For example, heavy metals can bind to the enzyme's active site instead of the substrate. They can also bind to the enzyme's allosteric site; in which case they

modify the enzyme's conformation. But in lower concentrations, thanks to pretreatment with activated carbon, the bacteria can grow and produce PHA, because they have no harmful effect [60]-[62].

3.7. Monomer Composition of PHA Products

The aim is to find out where our PHA produced in this study can be useful. The PHAs produced by *Cupriavidus necator* from molasses (untreated) and molasses pretreated (with sulfuric acid, enzyme and activated carbon), are composed of polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) (Table 4). The monomeric composition reflects the nature of the bacteria. The content of PHB is higher than PHV. For example, molasses pretreated with the enzyme (15%) at 96h gave 99.89 wt.% PHB and 0.11 wt.% PHV (Table 4). *Cupriavidus necator* is one of the most studied bacteria for PHB production. It can use different carbon sources, such as short-chain fatty acids, long-chain fatty acids and sugars, to synthesize mainly PHB [63] [64]. The various types of pretreatments did not influence the type of PHA produced by the bacteria. Monomeric composition also reflects the nature of the substrate [65] [66]. The results about monomeric composition of PHAs produced highlighted the use of the Embden-Meyerhof-Parnas pathway in the cytoplasm of the bacteria. This pathway breaks down glucose to produce pyruvate. The pyruvate thus formed is converted into acetyl-CoA by an enzyme complex called pyruvate dehydrogenase. Acetyl-CoA is then converted to PHA by three specific enzymes: 3-ketothiolase (phaA), acetoacetyl-CoA reductase (phaB) and PHA synthase (phaC) [67]. During biosynthesis, the PHAs accumulated are made up of monomers with the same chain length as the substrate. Or a length equal to one or more units 2 carbon atoms shorter. Moreover, monomers produced are PHB and PHV with respectively 4 and 5 carbons. We would like to remind molasses has mainly glucose and fructose, which are 6-carbon sugars [68].

Table 4. PHB and PHV content according to various pretreatment of molasses.

Source carbon	Harvest time (h)	PHB %	PHV %
Molasses	24	72.24	27.76
	48	78.59	21.41
	72	76.92	23.08
	96	84.80	15.20
H_2SO_4 5%	24	98.19	1.81
	48	99.38	0.62
	72	99.61	0.39
	96	99.41	0.59
H_2SO_4 10%	24	99.44	0.56
	48	99.60	0.40
	72	99.72	0.28
	96	99.44	0.56

Continued

H_2SO_4 15%	24	99.44	0.56
	48	99.67	0.33
	72	99.71	0.29
	96	99.63	0.37
EZ 3%	24	99.66	0.34
	48	99.78	0.22
	72	99.78	0.22
	96	99.81	0.19
EZ 10%	24	99.85	0.15
	48	99.91	0.09
	72	99.92	0.08
	96	99.88	0.12
EZ 15%	24	99.65	0.35
	48	99.92	0.08
	72	99.88	0.12
	96	99.89	0.11
AC 1.5%	24	80.61	19.39
	48	80.35	19.65
	72	77.95	22.05
	96	79.98	20.02
AC 13%	24	87.32	12.68
	48	98.30	1.70
	72	99.45	0.55
	96	99.20	0.80
AC 20%	24	78.12	21.88
	48	82.23	17.77
	72	88.80	11.20
	96	94.58	5.42

3.8. Comparison with Another Authors

Table 5 shows the comparison of PHA production by various bacterial utilizing cane molasses as carbon sources with different authors. [69] reported the highest PHA percentage (74.6 wt.%) in mixed microbial culture utilizing cane molasses as carbon source. The most recent study with [70] obtained 61.6 wt.% PHA with Bacillus thuringiensis HA1. In this study, the obtained PHA percentage (75.64 wt.%) is higher than recent study, but is so close to study, which reports the highest PHA percentage.

Table 5. Comparison of PHA production by various bacteria utilizing cane molasses as carbon sources.

Bacteria	Fermentation mode	Harvest time (h)	PHA (wt. %)	PHA (g/L)	References
Cupriavidus necator 11599	Rotary Shaker	96	75.64	7.59	This study
Mixed microbial culture	Bioreactor Batch	-	74.6	-	[69]
Bacillus thuringiensis HA1	-	36	61.6	-	[70]
Bacillus subtilis	Rotary Shaker	96	62.21	-	[71]
Escherichia coli	Rotary Shaker	96	58.7	-	[71]
Cupriavidus necator DSM 428	Rotary Shaker	-	24.33	1.41	[72]
Mixed microbial culture	Bioreactor Batch	-	57.5	-	[73]

Table 6. Comparison of PHA production by various bacteria and from various carbon sources.

Carbon source	Bacteria	Fermentation mode	Harvest time (h)	PHA (wt. %)	PHA (g/L)	References
Molasses	*Cupriavidus necator* 11599	Rotary Shaker	96	75.64	7.59	This study
Molasses	*Escherichia coli*	Bioreactor Batch	-	75.5	3.06	[74]
Glucose	*Saccharophagus degradans*	Rotary Shaker	-	22.4	-	[75]
		Bioreactor Fed Batch	-	52.8	-	
Volatile fatty acids	Mixed microbial culture	Bioreactor Batch	-	66.4	-	[76]
Cheese whey	*Haloferax mediterranei*	Bioreactor Batch	-	53	-	[77]
Olive mill wastewater	*Haloferax mediterranei*	Rotary Shaker	-	43	-	[78]
Styrene	*Pseudomonas putida* NBUS12	Rotary Shaker	-	32.49	-	[79]
Condensed Corn Solubles with Glycerol Water	*Pseudomonas putida* KT217	Bioreactor Fed Batch	85	31	8	[15]

Table 6 shows the comparison of PHA production by various bacteria and from various carbon sources. Compared to *Cupriavidus necator* (used in this study), *Escherichia coli* which also used molasses as a carbon source gives 75.5% approximately the same percentage of PHA obtained in this study [74]. However, the culture with this study was in a shake flask and the one with *Escherichia coli* was in a bioreactor. Since bioreactor culture is supposed to improve PHA production yield. Because of better control of parameters (temperature, pH, aeration, agitation, etc.) and the possibility of controlled feeding (fed-batch), unlike shake flasks [75]. Also, with *Saccharophagus degradans*, other bacteria, which used glucose (pure substrate) as carbon source, a PHA percentage of 22.4% and 52.8% was obtained with rotary shaker mode and bioreactor fed batch mode, respectively. These values are lower than those obtained in this study [75]. In addition, this study gives a higher percentage than that of the volatile fatty acid (66.4%) used as a carbon source to produce PHA by mixed culture [76]. Cheese whey, a complex substrate like molasses, was hydrolyzed into glucose and galactose, to facilitate its assimilation by *Haloferax mediterranei*. A percentage PHA of 53% was obtained; this value is also lower than that of this study [77]. The same bacteria, but with

olive mill wastewater as a carbon source, accumulated 43% of PHA, still lower than in this study [78]. The different recombinant strains of *Pseudomonas putida*, genetically modified to improve PHA production, gave a low PHA of 32.49% and 31% compared with this study, with styrene [79] and condensed corn soluble with glycerol water respectively [15].

4. Conclusion

Aiming to improve PHA production in the bacteria from molasses, 3 different pretreatments of molasses with acid, enzyme and activated carbon were applied. Results showed that the various pretreatments acted on sucrose and mineral concentration. The efficiency of pretreatments was evaluated by monitoring the evolution of bacterial growth and PHA production with pretreated and unpretreated molasses. The results showed that two of the pretreatments were able to hydrolyze sucrose (acid and enzyme). The other pretreatment (activated carbon) eliminates minerals. After these three pretreatments, the bacteria were able to accumulate a high percentage of PHA from molasses. Then, a study of the effect of the ratio (pretreatment/molasse) on PHA production showed that it is important to determine the suitable ratio. Ratios of pretreatment are important factors for enhancing PHA production. (acid/molasse), (enzyme/molasse) and (activated carbon/molasse) ratios that resulted adequate for PHA accumulation were (15:100), (15:100) and (20:100) attaining a maximum of 64.56, 75.64 and 58.14 wt.% PHA respectively. The monomer composition of the various PHAs obtained was determined. The results showed a PHA with a high PHB content and a small amount of PHV. The PHB produced can be used in packaging (especially food packaging) and biomedical applications. Further studies on the combination of pretreatments are needed to improve PHA production.

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Data Availability Statement

The datasets generated during the current study are available, from the corresponding author on request.

Consent to Participate

All authors agreed to participate in this work.

Consent to Publish

All authors agreed to this version for publication.

Authors' Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Oceanne Murielle Bohasset Mouho, Song Yan and Soro Doudjo. The first draft of the manuscript was written by Oceanne Murielle Bohasset Mouho. Affoué Tindo Sylvie Konan, Soro Doudjo, Kouassi Benjamin Yao, Patrick Drogui and Rajeshwar D Tyagi commented on previous versions of the manuscript. All authors read and approved of the final manuscript.

Conflicts of Interest

The authors have no relevant financial or non-financial interests to disclose.

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