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Cassava Peel Starch as a Raw Material for Polyhydroxyalkanoates Synthesis by *Cupriavidus necator*

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Abstract

The environmental problems caused by plastics of fossil origin are well known. To reduce harmful impact on the environment, bacterial-based plastics, such as polyhydroxyalkanoates (PHAs), are a promising solution. Microbial PHAs can be produced using abundant and inexpensive agricultural by-products as raw material. In this study, the potential use of *Cupriavidus necator* 11599 for the bioconversion of cassava starch into biodegradable PHAs was explored. Although *Cupriavidus necator* 11599 is a well-known PHA producer, it cannot grow directly on starch. Thus, acid hydrolysis was carried out on the starch extracted from cassava peels to obtain fermentable sugars. Optimal concentration of reducing sugars (RSs) was obtained by hydrolysis of cassava peel starch with sulfuric acid concentrations of 0.4 N and 0.6 N, at 95˚C and 4 h. The hydrolyzed starch was used for PHA production in Erlenmeyer flasks using reducing sugars (RSs) concentrations ranging from 10 g/L to 25 g/L. The best RS concentration 20 g/L and 25 g/L gave 85.13% ± 1.17% and 89.01% ± 2.49% of biomass PHA content and biomass concentrations of 8.18 g/L and 8.32 g/L, respectively in 48 hours. This research demonstrates that cassava peel starch as an inexpensive feedstock could be used for PHA production, paving the way for the use of other starchy materials to make bioplastics.

Keywords

Polyhydroxyalkanoate, *Cupriavidus necator*, Cassava Peels, Starch, Acid Hydrolysis

1. Introduction

Petroleum-derived plastics, such as polyethylene, polypropylene and polystyrene, are increasingly used in modern living and industrial applications [1]. Despite their mechanical properties, their persistence contributes to environmental pollution and increase in greenhouse gases [2]. To this end, it is therefore essential to find alternative resources that are sustainable and renewable. In recent years, many scientists have developed biodegradable composites as promising alternatives to synthetic plastics [1]. Among all bioplastics, polyhydroxyalkanoates (PHAs), a family of polyesters, show promise due to their physico-chemical properties like petroleum-derived plastics [3]. PHAs are biodegradable polyesters synthesized by different bacterial strains that store these polymers in intracellular pockets as energy reserves in response to excess carbon and nutrient-limited conditions [4]. PHAs can be exploited in a variety of applications, particularly in the pharmaceutical [5], agriculture, automotive, medicine [6] [7], textiles and packaging [8]. However, the production of PHAs on an industrial scale has higher manufacturing costs compared to plastics from petrochemicals [9]. This is mainly due to the high cost of the substrates, their production process, and the high cost of recovery reagents [10]. Due to these multiple applications, several industries have embarked on the production of PHAs and have started looking for ways to reduce production costs. Several methodologies and technologies have been tried and several substrates have been studied. Among the substrates used are starchy agricultural residues. Starch is the second most abundant carbon source, which is why it has been used as an economical carbon source in the production of a variety of value-added products, such as bioethanol, maltose syrup and others [11]. It offers desirable benefits, including its availability in nature, non-polluting effects, non-toxicity, renewable character and high production potential [12].

This work carried out focuses on the valorization of starchy agricultural residues as promising substrates to produce PHAs. Unfortunately, in nature, it is not possible to find a wild-type strain capable of both efficiently hydrolyzing a complex carbon source such as starch and accumulating PHAs in very high yields [13]. *Cupriavidus necator* 11599, one of the most promising PHA producers, can convert only simple monosaccharides, like glucose, into PHA. Thus, to transform starchy substrates into PHAs, the substrates must first be hydrolyzed, and the resulting sugars can be converted into PHAs by *Cupriavidus necator*. Pretreatments of starchy agricultural residues are expensive and energy-intensive. In this study, we explored the optimization of starch hydrolysis of cassava peels by varying temperature, time and acid concentration in order to obtain reducing sugars necessary for bacterial fermentation. We also examined the effect of the concentration of the carbonaceous substrate on the production of PHA. To the best of our knowledge, no previous research on cassava peel starch for production of value-added PHA is reported in literature.

The objective of this research is to develop an efficient and sustainable process to produce PHA from cassava waste as renewable raw material and environmental

impact reduction of petroleum-based plastics.

2. Materials and Methods

The chemicals used in this study were purchased from Sigma-Aldrich, ensuring high analytical quality that is suitable for laboratory use.

2.1. Microbial Culture

Cupriavidus necator 11599 was cultured and streaked on agar plates in mineral medium. The mineral medium is composed of (g/L): 6.00 $Na_2HPO_4{\cdot}12H_2O$; 2.4 KH_2PO_4; 1.00 NH_4Cl; 0.50 $MgSO_4{\cdot}7H_2O$; 0.01 $FeCl_3{\cdot}6H_2O$; 0.01 $CaCl_2{\cdot}6H_2O$; 20 Agar; 20 glucose. The $FeCl_3{\cdot}6H_2O$ and $CaCl_2{\cdot}6H_2O$ solutions were sterilized by filtration through a 0.2 µm polyethersulfone (PES) membrane filter. The KH_2PO_4 and Na_2HPO_4 solutions were autoclaved together; $MgSO_4$ and NH_4Cl were autoclaved separately at 121˚C for 15 min. These solutions were mixed aseptically after cooling. The average pH was maintained at 6.8. The plates were incubated at 30˚C ± 1˚C for 48 h and then stored at 4˚C for later use [4].

2.2. Cassava Peel Starch Extraction

The cassava was bought in a local market in Lomé, and then they were washed and peeled to collect the fresh cassava peelings. The raw starch was obtained by grinding the peelings with an excess of water in a blender. The mixture obtained was filtered and the filtrate was decanted. There is a liquid phase and a solid phase. The liquid phase (water) was discarded and the solid phase, which corresponds to the raw starch, was collected and dried at room temperature for 24 h and kept for later use. The equipment was sterilized by washing with an ethanol solution, following standard laboratory practices. The raw starch was stored at low temperatures (4˚C) to prevent degradation and maintain sample integrity until further use.

2.3. Alkaline Pretreatment

The alkaline pretreatment was carried out by introducing 100 g of starch into the Erlenmeyer flasks (1 L) containing a variable concentration of sodium hydroxide solution (0.1 - 0.6 N). Then, the mixtures were heated in a water bath for 4 h at 95˚C. The mixtures were filtered, and then the concentration of the reducing sugar was determined in the hydrolysates obtained (liquid fraction).

2.4. Raw Starch Acid Pretreatment

The acid pretreatment was carried out according to the method of [14]. To perform the cassava peal starch hydrolysis, 100 g of raw starch was introduced into Erlenmeyer flasks (1 L) containing a variable concentration of sulfuric acid solution (0.025 - 0.8 N). Then, the mixtures were heated in a water bath for 4 h at 95˚C. The mixtures were filtered, and then the concentration of the reducing sugar was determined on the hydrolysates (liquid fraction) obtained. To optimize the concentration

of sulfuric acid, the temperature and the reaction time for the peal starch hydrolysis were made at 50˚C, 70˚C, 80˚C, 95˚C for 0.5 h; 1 h; 2 h; 3 h.; 4 h. The hydrolysates were stored at low temperatures (4˚C) to prevent degradation and maintain sample integrity until further use.

2.5. Enzymatic Pretreatment of Starch

The enzymatic pretreatment was carried out according to the method of [15]. Raw starch hydrolysis (30 g/100 mL) was carried out following the steps: First, liquefaction at 90˚C with pH 6.5 for 45 minutes using 4 kilo novo units (KNU (S)/g starch) of commercial amylases derived from *Bacillus licheniformis*. Then, saccharification was carried out at 60˚C with a pH of 4 for 60 minutes using 6 amyloglucosidase units (AGU/g starch) of commercial glucoamylase derived from *Aspergillus niger*. KNU (S) is defined as the amount of enzyme required to break down 5.26 g of starch per hour, according to the novozyme standard method for the determination of α-amylases. As for the AGU, it is defined as the quantity of enzyme capable of hydrolyzing 1 mol of maltose per minute at 37˚C and a pH of 4.3. The hydrolysate was filtered and the resulting filtrate containing reducing sugar was used as a carbon source for the production of PHAs (polyhydroxyalkanoates).

2.6. PHA Production

2.6.1. Preculture 1

Loops of *Cupriavidus necator* from plates were inoculated into Erlenmeyer flasks containing sterilized medium (100 mL). Preculture medium 1 was composed of (g/L): 6.00 $Na_2HPO_4{\cdot}12H_2O$; 2.4 KH_2PO_4; 1.00 NH_4Cl; 0.50 $MgSO_4{\cdot}7H_2O$; 0.01 $FeCl_3{\cdot}6H_2O$; 0.01 $CaCl_2{\cdot}6H_2O$; 20 glucose. The solution of $FeCl_3{\cdot}6H_2O$ and $CaCl_2{\cdot}6H_2O$ was sterilized by filtration through a 0.2 µm polyethersulfone (PES) membrane filter. The KH_2PO_4 and Na_2HPO_4 solutions were autoclaved together; glucose, $MgSO_4$ and NH_4Cl were autoclaved separately at 121˚C for 15 min. These solutions were mixed aseptically after cooling. The average pH was maintained at 6.8. The flasks were incubated on a rotary shaker at 300 rpm at 30˚C for 24 h.

2.6.2. Preculture 2

Actively growing cells from preculture 1 (10% v/v) were used as inoculum for preculture 2. The composition of the mineral medium is the same as that of preculture 1. Starch hydrolysate (10 g/L, 15 g/L, 20 g/L, 25 g/L) was used as a carbon source. The pH of the hydrolysate was adjusted with a sodium hydroxide solution (4 N) to maintain pH of the medium at 6.8. The KH_2PO_4 and Na_2HPO_4 solutions were autoclaved together; $MgSO_4$ and NH_4Cl were autoclaved separately along with the starch hydrolysate, each at 121˚C for 15 min. After cooling, the minerals and the carbonaceous substrate were mixed aseptically and inoculated. The flasks were incubated on a rotary shaker at 200 rpm at 30˚C for 24 h.

2.6.3. Production of PHAs in Erlenmeyer Flasks

10% of the volume of preculture 2 was transferred to 300 mL of production

medium in a 2 L Erlenmeyer flask. The composition of the production media was the same as the second preculture medium 2 and the concentration of reducing sugars varied from 10 to 25 g/L. The pH was maintained at 6.8 by adding sodium hydroxide solution (4 N) to the media. Flasks were incubated in a rotary incubator at 30˚C; the stirring speed was maintained at 200 rpm and the fermentation was carried out for 96 h. Samples were taken every 24 hours.

2.7. Analysis Methods

2.7.1. Suspended Solids Analysis and PHA Extraction

The concentration of biomass or suspended solids (SSs) was measured according to the standard method. To determine the biomass concentration and the PHA content in the cells, the samples (20 mL) were centrifuged, and the cells were washed with distilled water. Biomass concentration is expressed as cell dry weight. The PHA content of the cells was analyzed by gas chromatography (Agilent Technologies, model 7890B flame ionization detector (FID)) using the standard methanol and chloroform extraction method [16]. P (3HB-co-3HV) composed of 8% by moles of 3 HV (Polyhydroxyvalerate) and 92% moles of 3HB (polyhydroxybutyrate), was used as standard.

2.7.2. Determination of Reducing Sugars

The reducing sugars of the supernatants recovered after centrifugation of the samples were determined. The method used is the DNS (3,5-dinitrosalicylic) method [17].

3. Results and Discussion

3.1. Chemical Characterization of Raw Starch

In this study, raw starch extracted from cassava peels was characterized to determine its physicochemical composition. The results in **Table 1** show that the raw starch powder consisted of a low level of fermentable sugars. As a result, hydrolysis was an important step in this study because it made possible to obtain fermentable sugars to produce PHAs. Trace elements were quantified using inductively coupled plasma mass spectrometry (ICP-MS), which enables precise measurement of trace elements.

Table 1. Chemical characterization of raw starch.

Composition	Concentration	Composition	Concentration
Xylose (mg/L)	-	Potassium (mg/kg)	732
Fructose (mg/L)	-	Magnesium (mg/kg)	33.5
Glucose (mg/L)	-	Manganese (mg/kg)	0.31
Sucrose (mg/L)	-	Sodium (mg/kg)	60.1
reducing sugar (g/L)	-	Phosphorus (mg/kg)	75
Total nitrogen (mg/kg)	0.030	Lead (mg/kg)	-
Aluminum (mg/kg)	17.7	Sulfur (mg/kg)	24

Continued

Boron (mg/kg)	-	Selenium (mg/kg)	-
Barium (mg/kg)	0.87	Silicon (mg/kg)	29
Calcium (mg/kg)	90.1	Tin (mg/kg)	-
Cadmium (mg/kg)	-	Strontium (mg/kg)	0.82
Chromium (mg/kg)	-	Titanium (mg/kg)	0.21
Copper (mg/kg)	-	Vanadium (mg/kg)	-
Iron (mg/kg)	20	Zinc (mg/kg)	0.9

3.2. Results of Enzymatic and Alkaline Pretreatments

The results of the alkaline pretreatment presented in Table 2 show that the hydrolysates obtained have a concentration of reducing sugars varying from 0.74 g/L to 1.22 g/L. As for the enzymatic hydrolysis of starch, the hydrolysate obtained contained a concentration of 191 g/L of reducing sugars. Due to the high costs associated with enzymes, an acid pretreatment approach was chosen for the continuation of this study.

Table 2. Alkaline pretreatment with an initial concentration of raw starch of 100 g/L.

Solution of sodium hydroxide (N)	0.1	0.3	0.4	0.6
Reducing sugar (g/L)	0.74	0.77	0.96	1.22

3.3. Acid Pretreatment

The hydrolysis of the starch was carried out in a water bath with a solid load of 100 g/L. The operational variables were temperature (50˚C - 95˚C), sulfuric acid concentration (0.2 - 0.6 N), reaction time (0.5 - 4 h). The reducing sugar concentrations after acid hydrolysis under different treatment conditions are shown in Table 3 and Figure 1.

3.3.1. Effect of Acid Concentration on Reducing Sugar Concentration

The results obtained demonstrated that the reducing sugar concentration of acid hydrolysates increases with the concentration of sulfuric acid used during the hydrolysis of starch from cassava peels. This indicates that acidity plays a crucial role in the breakdown of starch into reducing sugars. Figure 1 clearly shows a positive correlation between sulfuric acid concentration and reducing sugar concentration. The best results are observed with sulfuric acid concentrations of 0.4 N and 0.6 N, where the syrup obtained exhibited the highest reducing sugar concentration (57.56 g/L and 58.67 g/L).

However, it is important to note that the reducing sugar concentration did not increase with the acid concentration of more than 0.6 N. In fact, a reduction in the concentration of reducing sugar is observed further increase in the acid concentration. This decrease can be explained by several factors. First, higher sulfuric acid concentration can lead to undesirable side reactions, such as the breakdown of reducing sugars into non-reducing products (such as sucrose). In addition,

excess acidity can cause thermal degradation of reducing sugars, as well as their decay in acid hydrolysates. Taking into account environmental considerations (the lowest acid concentration that maximizes the reducing sugar concentration) and the similarity of reducing sugar concentrations, the sulfuric acid solution with a concentration of 0.4 N seems to be the most suitable. This concentration makes it possible to obtain the highest concentration of reducing sugar while minimizing the use of sulfuric acid. Using a higher concentration, such as 0.6 N, could result in additional effects without providing significant benefits in terms of reducing sugar concentrations.

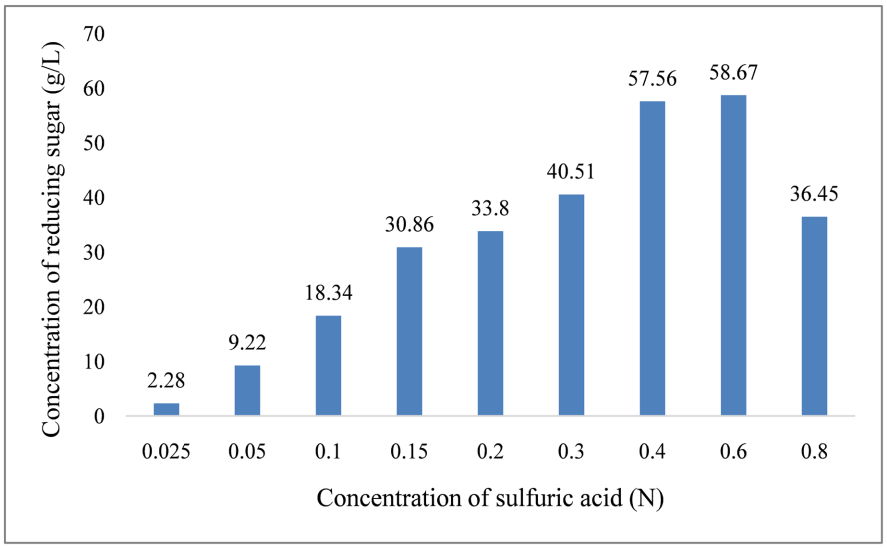

Figure 1. Evolution of the concentration of reducing sugar as a function of the concentration of sulfuric acid at 95˚C for 4 h.

3.3.2. Effect of Time and Temperature on Reducing Sugar Concentration

The effect of time and temperature on the reducing sugar concentration of the starch hydrolyzate obtained from acid hydrolysis is presented in **Table 3**.

An increase in the concentration of reducing sugar is observed with the increase in the reaction time irrespective of temperature and the acid strength. For example, for the hydrolysis conducted with 0.6 N sulfuric acid at 95˚C, the concentration of reducing sugar increases from 1.49 g/L to 33.8 g/L with reaction time. This indicates that starch hydrolysis continues gradually over time, releasing more reducing sugars. About the effect of temperature, there is also a significant influence on the concentration of reducing sugar. Overall, an increase in temperature favors an increase in reducing sugar. For example, for a sulfuric acid concentration of 0.4 N, the reducing sugar concentration at 50˚C is 0.47 g/L, while it reaches 2.53 g/L at 70˚C and 58.67 g/L at 95˚C, after 4 h of reaction (**Table 3**). This suggests that higher temperatures accelerate starch hydrolysis, leading to greater release of reducing sugars. These results are similar to those of Chavan *et al.* [18] who worked on optimizing the acid hydrolysis of pure starch. They varied the temperature from 50˚C to 110˚C and set the reaction time to 60 min and sulfuric acid concentration

was 1% (v/v). The concentration of reducing sugar increased from 1.37 g/L to 1.91 g/L depending on the evolution of the temperature. Similarly, they varied the time from 0 to 80 min and the concentration of reducing sugars increased from 1 to 1.9 g/L. These results highlight the importance of reaction time and temperature in the acid hydrolysis of raw starch from cassava peels. An increase in reaction time allows a complete conversion of starch into reducing sugars, while an increase in temperature accelerates the hydrolysis process [19] [20]. It should be noted that the optimization of the reaction conditions must consider other factors such as the efficiency of the conversion of starch into reducing sugars, the cost and the environmental impact of the reagents used. However, the results obtained for longer reaction times and higher temperatures may be favorable for increasing the concentration of reducing sugar.

In view of these results, it can be deduced that the optimal conditions for carrying out the hydrolysis of aqueous solutions of cassava peelings starches are 95˚C for 4 h.

Table 3. Evolution of reducing sugar concentration as a function of temperature and reaction time with an initial concentration of raw starch of 100 g/L.

Time (h)	Concentration of reducing sugar of the hydrolysate of starch (g/L)								
	H_2SO_4 (0.2 N)			H_2SO_4 (0.4 N)			H_2SO_4 (0.6 N)		
	50˚C	70˚C	95˚C	50˚C	70˚C	95˚C	50˚C	70˚C	95˚C
0.5	0.21	0.05	1.49	1.05	2.34	9.02	0.47	2.39	13.54
1	0.04	0.09	2.79	1.17	2.54	13.95	0.59	2.56	22.29
2	0.05	0.30	2.38	1.35	2.61	24.18	1.06	2.44	28.53
3	0.11	0.31	3.03	1.12	2.41	29.73	1.44	2.09	29.81
4	0.10	1.51	33.80	1.60	2.53	57.56	1.50	2.55	58.67

3.3.3. Chemical Characterization of the Raw Starch Hydrolysate Obtained after the Acid Pre-Treatment

The results of the chemical characterization of the starch hydrolysate are presented in Table 4. There is a high concentration of glucose (47 g/L) present in the hydrolysate, while the concentration of minerals and nitrogen were weak. The presence of monosaccharide sugars such as glucose is advantageous because it allows easy and rapid assimilation by PHA-producing bacterial cells [21]. In addition, the concentration of glucose/galactose in the enzymatic hydrolysate (Table 5) is 190 g/L and the presence of fructose is noted.

Table 4. Chemical composition of raw starch hydrolysate with an initial concentration of raw starch of 100 g/L.

Composition	Concentration	Composition	Concentration
Xylose (mg/L)	-	Potassium (mg/L)	68.4
Fructose (mg/L)	-	Magnesium (mg/L)	6.49
Glucose (mg/L)	47,000	Manganese (mg/L)	0.213
Lactose (mg/L)	12,000	Sodium (mg/L)	3075

Continued

Sucrose (mg/L)	-	Phosphorus (mg/L)	9.49
Reducing sugar (g/L)	58,000	Lead (mg/L)	0.019
Total nitrogen (mg/L)	0.807	Sulfur (mg/L)	63.8
Aluminum (mg/L)	3.31	Selenium (mg/L)	0.08
Boron (mg/L)	0.36	Silicon (mg/L)	4.86
Barium (mg/L)	0.131	Tin (mg/L)	0.034
Calcium (mg/L)	22.8	Strontium (mg/L)	0.227
Cadmium (mg/L)	0.0007	Titanium (mg/L)	0.051
Chromium (mg/L)	0.006	Vanadium (mg/L)	0.005
Copper (mg/L)	0.027	Zinc (mg/L)	0.143
Iron (mg/L)	1.81		

Table 5. Different sugars present in the enzymatic hydrolysate.

Sugar	Concentration (mg/L)
Glucose/galactose	190,000
Fructose	1100
Lactose	540
Reducing sugar	191,000
Sucrose	-
Xylose	-

3.4. Production of PHAs

As a reminder, experiments were carried out in Erlenmeyer flasks under agitation to produce PHAs. The flasks were incubated at 30˚C and shaken at 200 rpm for 96 h. The effect of the concentration of the carbonaceous substrate on cell growth and the production of PHAs was studied. The carbonaceous substrate concentration varied from 10 to 25 g/L and samples were taken every 24 h. The data obtained are presented in **Table 6**.

3.4.1. Effect of Reducing Sugar Concentration on Cell Biomass

Cell growth and PHAs accumulation peaked after 48 h regardless of carbon substrate concentration (**Figure 2**). There was a slight decrease in the accumulation of PHAs after 72 h (**Figure 2**). This decrease is likely due to substrate depletion and the formation of intracellular by-products that caused the inhibition [13]. The consumption of the carbonaceous substrate in the fermented broths is illustrated in **Figure 3**. This decreases rapidly from 0 h to 48 h, hence the exhaustion of the carbonaceous substrate. The maximum concentration of cell biomass is 9.22 ± 0.51 g/L.

However, the biomass concentration (suspended solids) increased from 0 h to 96 h according to the results in **Table 6** and **Table 7**. In addition, the increase in the carbon source leads to an increase in the biomass content. **Figure 2** shows that

with 20 g/L and 25 g/L of carbon sources, the maximum biomass content was respectively 9.22 ± 0.51 and 8.96 ± 0.78 g/L at 96 h.

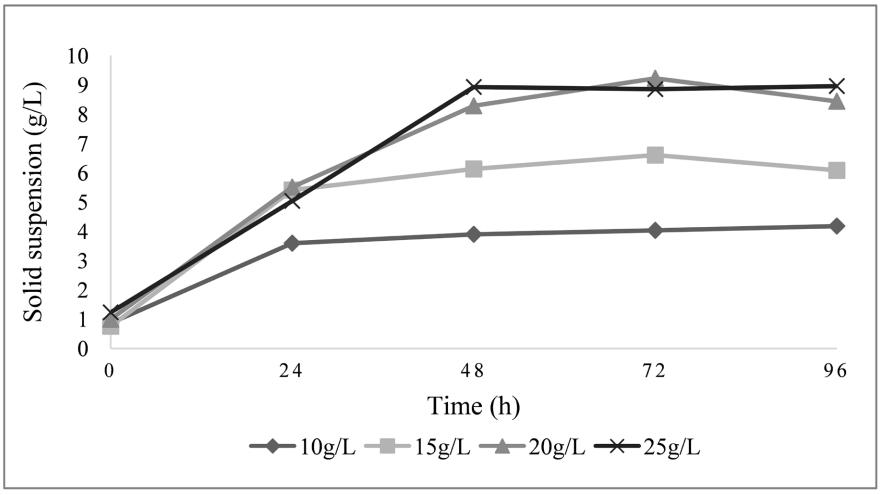

Figure 2. Evolution of the concentration of suspended solids (biomass) as a function of time and of the concentration of the starch hydrolysate obtained from an initial concentration of raw starch of 100 g/L.

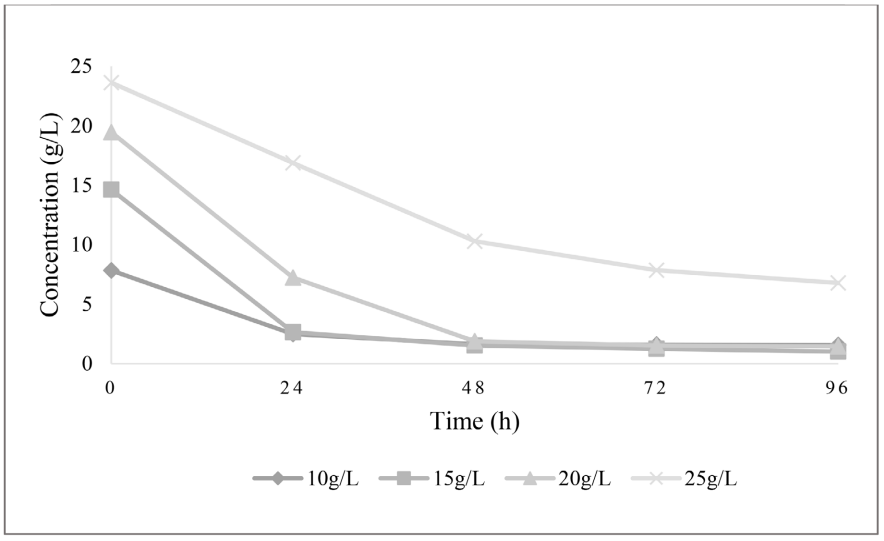

Figure 3. Evolution of reducing sugar consumption as a function of time.

Table 6. Summary of the results of the production of PHAs, reducing sugar (RS) solid in suspension (SS) with the acid hydrolysate obtained from an initial concentration of raw starch of 100 g/L.

Biomass concentration	Parameters	Time (h)				
		0	24	48	72	96
10 g/L	RS (g/L)	7.91 ± 0.06	3.34 ± 0.85	2.81 ± 1.18	2.70 ± 1.10	2.55 ± 0.99
	SS (g/L)	0.70 ± 0.17	2.82 ± 0.77	3.01 ± 0.89	3.25 ± 0.78	3.40 ± 0.78
	PHA (%)	0.00 ± 0.00	21.04 ± 6.87	20.53 ± 8.89	18.20 ± 7.99	18.63 ± 10.34
	PHA (g/L)	0.00 ± 0.00	0.59 ± 0.18	0.62 ± 0.18	0.61 ± 0.22	0.62 ± 0.21

Continued

15 g/L	RS (g/L)	14.63 ± 0.77	2.65 ± 0.31	1.53 ± 0.30	1.26 ± 0.29	1.02 ± 0.05
	SS (g/L)	0.76 ± 0.16	5.41 ± 0.05	6.13 ± 0.04	6.60 ± 0.18	6.08 ± 0.16
	PHA (%)	0.00 ± 0.00	58.31 ± 1.13	65.78 ± 2.45	63.19 ± 3.66	61.91 ± 1 .27
	PHA (g/L)	0.00 ± 0.00	3.15 ± 0.03	4.03 ± 0.12	4.17 ± 0.13	3.77 ± 0.18
20 g/L	RS (g/L)	17.46 ± 0.17	7.24 ± 0.19	1.89 ± 0.02	1.52 ± 0.17	1.46 ± 0.11
	SS (g/L)	0.99 ± 0.17	5.51 ± 0.18	8.28 ± 0.10	8.44 ± 0.38	9.22 ± 0.51
	PHA (%)	0.00 ± 0.00	58.73 ± 0.57	85.13 ± 1.17	83.55 ± 1.77	71.69 ± 0.90
	PHA (g/L)	0.00 ± 0.00	3.24 ± 0.01	7.05 ± 0.88	7.71 ± 0.30	6.05 ± 0.19
25 g/L	RS (g/L)	23.63 ± 0.73	16.88 ± 1.52	10.29 ± 2.27	7.86 ± 1.61	6.79 ± 1.27
	SS (g/L)	1.23 ± 0.35	5.04 ± 0.56	8.93 ± 0.60	8.85 ± 1.02	8.96 ± 0.78
	PHA (%)	0.00 ± 0.00	71.07 ± 0.02	89.01 ± 2.49	82.62 ± 1.81	81.19 ± 0.16
	PHA (g/L)	0.00 ± 0.00	3.58 ± 0.30	7.95 ± 0.07	7.31 ± 0.44	7.27± 0.00

Table 7. Results of the production of PHAs, reducing sugar (RS), suspended solids (SS) with the enzymatic hydrolysate.

Biomass concentration	Parameters	Time (h)				
		0	24	48	72	96
10 g/L	RS (g/L)	13.74	3.00	0.90	0.75	1.23
	SS (g/L)	0.60	5.97	7.24	7.76	7.38
	PHA (%)	0.00	19.43	16.95	13.71	13.63
	PHA (g/L)	0.0	1.2	1.2	1.1	1.0
15 g/L	RS (g/L)	12.17	1.12	0.93	0.4	0.25
	SS (g/L)	0.26	2.98	2.67	2.83	2.85
	PHA (%)	0.00	74.20	59.37	96.66	94.99
	PHA (g/L)	0.0	2.2	1.6	2.7	2.7
20 g/L	RS (g/L)	17.41	2.47	1.39	1.35	1.15
	SS (g/L)	0.84	5.09	7.75	9.79	8.31
	PHA (%)	0.00	68.22	65.41	85.73	82.42
	PHA (g/L)	0.00	3.47	5.07	8.40	6.85
25 g/L	RS (g/L)	23.90	4.53	1.52	1.09	0.34
	SS (g/L)	0.60	4.97	9.54	9.69	8.68
	PHA (%)	0.00	62.81	-	61.54	76.33
	PHA (g/L)	0.00	3.12	-	5.96	6.63

3.4.2. Effect of Reducing Sugar Concentration of Acid Hydrolysate on PHA Yield

The PHAs from the cell biomass were extracted by solvent extraction and the PHA concentration was calculated in g/L as shown in Figure 4. The PHA concentration varied for each concentration of the carbon source studied. The bacteria grew and produced PHAs on all different concentrations of the carbon source used. After 24 h of incubation, the biomass started to increase and reached its maximum value on the second day of incubation in all samples, although the daily increase was

different for the four concentrations of the carbon source. After 48 h, PHA yields decrease slightly. Maximum PHA yields were observed with carbon source concentrations of 20 and 25 g/L, reaching 85.13% ± 1.17% and 89.01% ± 2.49%, respectively. In addition, the yield of PHA increased as the concentration of carbon source increased from 10 to 25 g/L (**Figure 5**). These results indicate that the concentration of the carbon source has a positive effect on the production of PHA.

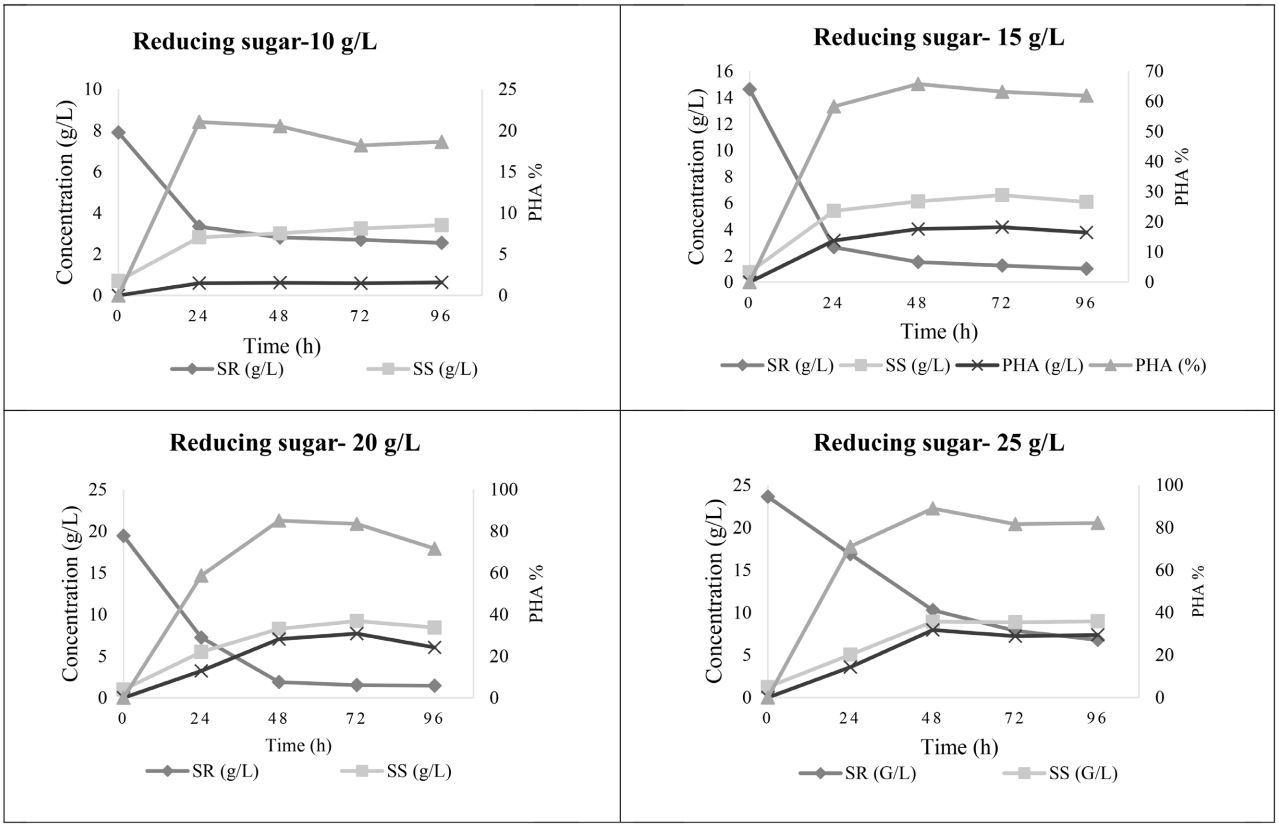

Figure 4. Effect of the concentration of the carbon source on the production of PHA.

Comparing these results to the study by [22], which used frying oil as a carbon source, the PHA concentrations obtained are significantly higher. They obtained PHA concentrations of 1.2 g/L in 72 h and 0.9 g/L in 48 h with 20 g/L of used frying oil. However, in this study, the concentration of PHA is 7.71 ± 0.30 g/L with 20 g/L of hydrolyzed starch in 72 h and 7.05 ± 0.88 g/L in 48 h (**Figure 6**). This significant difference could be explained by the fact that hydrolyzed starch is a more easily metabolizable carbon source for *Cupriavidus necator*. In addition, hydrolyzed starch may contain mineral salts and other growth factors that facilitate the metabolic activity of *Cupriavidus necator* thus contributing to high PHA production [23].

Additionally, different bacterial strains (*Cupriavidus* sp. *KKU*38, *Escherichia coli SKB*99, *Bacillus megaterium* respectively) and starch sources produce PHA [15] [24] [25]. PHA yields were 57.4%, 29.7%, and 61.6% respectively. The production

of PHAs using agro-industrial residues as carbon sources has also been studied by [15] [26]. They evaluated seven different substrates, such as wheat bran, potato starch, sesame cake, peanut cake, cassava powder, jackfruit seed powder, and maize flour. Substrates were hydrolyzed using commercial enzymes, to select the best substrate for PHA production. Jackfruit seed powder allowed maximum PHA production under submerged fermentation using *Bacillus sphaericus* (19%).

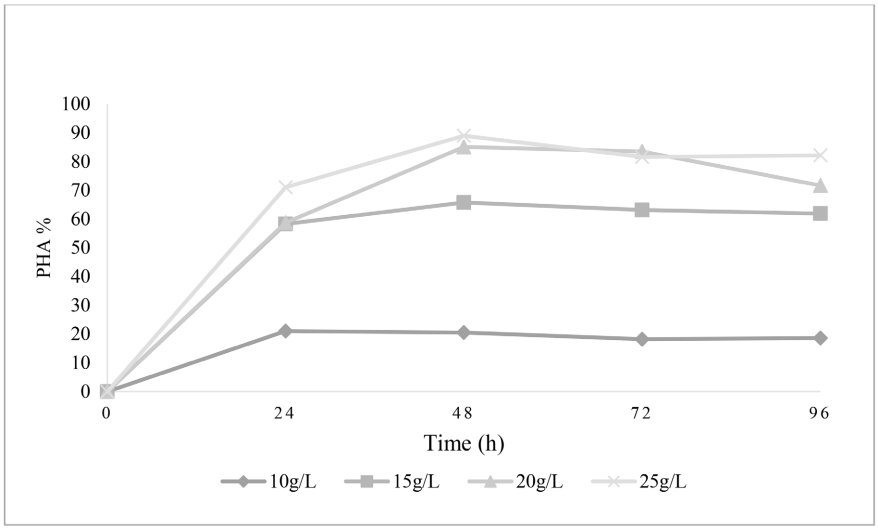

Figure 5. PHA content as a function of time and starch hydrolysate concentration.

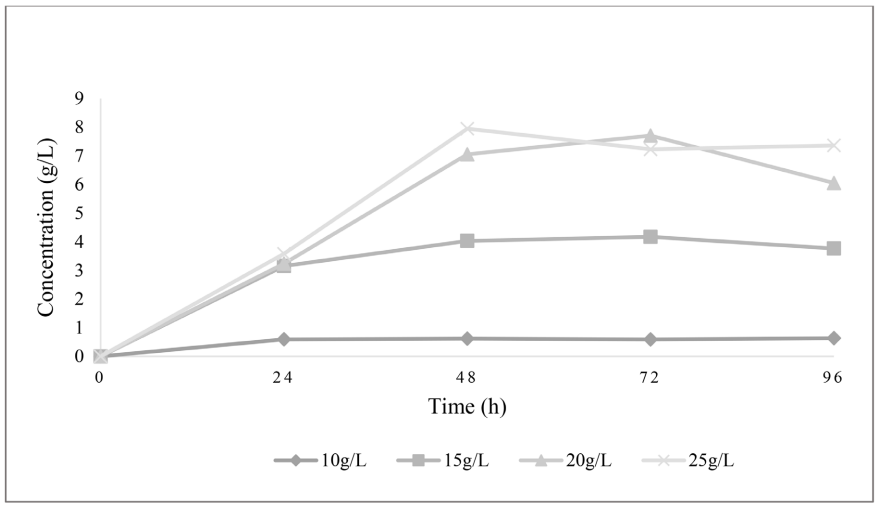

Figure 6. PHA concentration as a function of time and starch hydrolysate concentration.

3.4.3. Polyhydroxybutyrate (PHB) and Polyhydroxyvalerate (PHV) Copolymers

The analysis of the PHAs made it possible to determine the content of the various copolymers. The results in **Table 8** and **Table 9** show that the content of PHB and PHV varies depending on the concentration of the type of starch hydrolysate. The higher the concentration of starch hydrolysate, the higher the percentages of PHB

and PHV. This could be due to increased substrate availability for polymer synthesis at higher concentrations.

Table 8. PHB and PHV content obtained with the acid hydrolysate.

Hydrolyzed starch	Time (h)	PHB (%)	PHV (%)
10 g/L	24	93.39	6.60
	48	96.07	3.92
	72	94.27	5.73
	96	92.90	7.10
15 g/L	24	99.03	0.56
	48	99.04	0.96
	72	99.05	0.95
	96	99.04	0.96
20 g/L	24	99.34	0.66
	48	99.51	0.49
	72	99.45	0.55
	96	99.51	0.49
25 g/L	24	99.15	0.84
	48	99.60	0.40
	72	99.13	0.88
	96	99.40	0.44

Table 9. PHB and PHV content with enzyme hydrolysate.

Hydrolyzed starch	Time (h)	PHB (%)	PHV (%)
10 g/L	24	95.19	2.25
	48	96.69	3.28
	72	97.28	2.72
	96	97.72	4.81
15 g/L	24	99.11	0.09
	48	99.09	0.91
	72	99.07	0.93
	96	99.07	0.93
20 g/L	24	99.09	0.91
	48	99.51	0.49
	72	99.49	0.51
	96	99.43	0.57
25 g/L	24	99.03	0.97
	48	-	-
	72	99.63	0.38
	96	99.53	0.47

4. Conclusion

This study explored the use of starch extracted from cassava peels as a carbon source for the production of PHAs. Three hydrolysis methods were applied to the starch: an alkaline hydrolysis, which gave a hydrolysate containing a maximum of 1.22 g/L of reducing sugar, an enzymatic hydrolysis, which produced a hydrolysate containing 191 g/L of reducing sugar, and acid hydrolysis using a sulfuric acid solution at a specific concentration of 0.4 N, at a temperature of 95˚C for 4 h, leading to a high concentration of reducing sugar (57.56 g/L). Due to the inefficiency of alkaline hydrolysis and the high price of enzymes, acid hydrolysis was used to produce the reducing sugars necessary for fermentation. The production of PHAs from acid starch hydrolysate showed maximum accumulation after 48 h of fermentation, independent of the initial concentration of the carbonaceous substrate. However, a slight decrease in PHA accumulation was observed after 72 h. Rapid consumption of the reducing sugar resulted in its depletion at 48 h, with a maximum cellular biomass concentration of 9.22 ± 0.51 g/L. Optimum Erlenmeyer fermentation conditions were obtained with carbon source concentrations of 20 g/L and 25 g/L, thus achieving a yield of 89.01% ± 2.49% PHA. Some challenges encountered include the rapid depletion of reducing sugars, which limited sustained PHA production beyond 48 hours. Additionally, the use of sulfuric acid for hydrolysis may pose environmental considerations for scaling up the process. Future studies could explore more environmentally friendly hydrolysis methods or alternative nutrient supplementation strategies to sustain PHA production over extended fermentation periods. The use of cassava peel starch as a substrate for PHA synthesis holds potential for applications in biodegradable plastics, especially in regions with cassava production, supporting waste valorization and sustainable material development. Statistical analysis was conducted on the data, confirming that the observed PHA yields were statistically significant, with minimal variability between replicates.

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Data Availability Statement

The raw/processed data required to reproduce these findings can be shared on demand.

Consent to Participate

All authors agreed to participate in this work.

Consent to Publish

All authors agreed to this version for publication.

Authors' Contributions

Ida Diribissakou: Conceptualization, methodology, investigation, formal analysis, writing—original draft, and writing—review & editing. **Song Yan**: Conceptualization, methodology, writing—review & editing, and supervision. **Magnoudéwa Bassaï Bodjona**: Supervision and conceptualization, methodology, investigation, formal analysis, and original draft & editing. **Gado Tchangbedji**: Supervision. **Julien G. Mahy**: Conceptualization, formal analysis, and writing—original draft. **Patrick Drogui R. D. Tyagi** and **Benjamin K. Yao**: Conceptualization, methodology, writing—review & editing, investigation, formal analysis, supervision, funding acquisition, and project administration.

Conflicts of Interest

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

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