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BIOTECHNOLOGY AND INDUSTRIAL MICROBIOLOGY - RESEARCH PAPER

Fumaric acid production by Rhizopus species from acid hydrolysate of oil palm empty fruit bunches

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Abstract

The hemicellulosic fraction of lignocellulosic biomass is a very important material, due to the significant concentration of pentoses present in its composition and that can be used sustainably in biotechnological processes such as the production of fumaric acid. Research efforts are currently being promoted for the proper disposal and valorization of empty fruit bunches (EFB) from oil palm. In this work, seventeen Rhizopus species were evaluated in a fermentation medium with EFB hydrolyzate, without detoxification, as a carbon source for fumaric acid production. *Rhizopus circicans* 1475 and Rhizopus 3271 achieved productions of 5.65 $g.L^{-1}$ and 5.25 $g.L^{-1}$ of fumaric acid at 30 °C, 120 rpm for 96 h, respectively. The percentage of consumed sugars, mainly pentoses, was 24.88% and 34.02% for *R. circicans* 1475 and R 3271, respectively. Soy peptone and ammonium sulfate were evaluated as nitrogen sources, where soy peptone stimulated the formation of biomass pellets while ammonium sulfate produced mycelia and clamps.

Keywords *Rhizopus circicans* · Acid treatment · EFB · Pentoses · Fumaric acid

Introduction

Elaeis guineensis is a tropical plant that grows in highly moist warm weathers. The importance of this plant relies on oil production, which is used in the biodiesel production [1, 2] Indonesia is the main producer of crude palm oil, responsible for 60% of world production [3] and in South America stand up countries like Brazil, Colombia and Ecuador for having the largest palm plantations. This industry generates large amounts of wastes, such as oil palm empty fruit bunches (EFB) that are considered an environmental threat. EFB is mainly composed of approximately 39.0 ± 1.7% cellulose, 23.1 ± 1.2% hemicellulose and 20.1 ± 0.1% lignin [4]. The hemicellulose contained in the residue can be chemically hydrolyzed by dilute acid pretreatment, releasing sugars in the hydrolysate, mainly composed of pentoses like xylose and arabinose [5]. These pentoses could be biotechnologically used in fermentation processes to obtain organic acids of industrial interest.

Organic acids are low molecules weight that contain carboxyl groups. They are biologically important because they participate by interrelating in the main metabolic pathways of microorganisms. There are organic acids of industrial importance that are classified as safe (GRAS), among

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One-Sentence Summary: This investigation shows the production of fumaric acid by Rhizopus, under submerged fermentation using pentoses from EFB acid hydrolysates without detoxification.

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which acetic, propionic, lactic, citric and fumaric acids stand out [6]. Fumaric acid are considered important building in biopolymers construction [7]. Recently, European markets have regulated the entry of synthetic organic acids, favoring the naturally obtained ones. For this reason, it is imperative to generate environmental and friendly alternatives to obtain organic acids by microbial fermentation [8, 9]. In this line, it is expected to create new markets in the upcoming years [10]

Fumaric acid is an organic dicarboxylic acid that has the potential to make biopolymers and biodegradable resins [11]. It is produced by two routes, the petrochemical route that uses the isomerization of maleic acid and those generated by the microbial fermentation route [12] So far, chemical synthesis is the most profitable way of its production, compared to an 85%(w/w) yield generated by microbial fermentation [11] These properties are important for lifting a chemical platform. In addition, it is important as an input in food because it inhibits the growth of microorganisms due to its high acidity acting like a preservative and acidulent, the latter acting like flavour enhancing improvement, which differentiate fumaric acid from other more general purpose acidulates, such as citric acid and phosphoric acid [12, 13] It is also a valuable intermediate product for the production of L-malic acid and L-aspartic acid, which when polymerized can be applied in biomedical and pharmaceutical tasks [14].

Fungi of the genus Rhizopus have been reported to produce organic acids. They are capable to produce fumaric, lactic, malic acid, and even acid mixtures such as succinic and oxalic acid [15, 16]. These microorganisms can uptake sugars from lignocellulosic biomass hydrolysates and grow in the presence of microbial inhibitors in concentrations that other microorganisms could not survived [17]. Rhizopus, has been reported as a producer of tanases [18, 19] so it seems to be a good potential fungus to metabolize pentoses contained in lignocellulosic hydrolysates. The most reported species that produce fumaric acid are *R. oryzae*, *R. formosa*, *R. arrhizus* and *R. delemar* [20–22].

Fumaric acid has been produced by chemical synthesis and by microbial fermentation, in fact, microbial fermentation was initially relegated by chemical synthesis due to its low yields. The commercialization of organic acids increasingly requires greater quality criteria to be accepted by international markets, this leads to them being produced in an environmentally friendly manner [23]. In this sense, the microbial production of fumaric acid through microbial fermentation becomes relevant. Despite having previous experiences regarding microbial fermentation using agroindustrial waste, it is important to highlight EFB since it is one of the parts with the highest amount of sugars of the plant (part of this work) that can be available for microbial fermentation. The current challenges are to improve microbial fermentation optimization processes through metabolic engineering, genetic engineering and gene editing. Through these tools, the production of fumaric acid through microbial fermentation can be made profitable.

The objective of this research was to evaluate the production of fumaric acid by 17 different Rhizopus species, using the pentose-rich hydrolysate recovered from dilute acid pretreatment of EFB, an agricultural residue largely produced in the palm oil industry.

Materials and methods

Dilute acid pretreatment of EFB

The empty fruit bunches (EFB) from oil palm were obtained from the Vale Company (Mojú, Pará, Brazil). They were dried at 80 °C for 24 h and knife-milled to obtain a particle size in the range of 0.35—0.45 mm. EFB was mixed with a 1.5% sulfuric acid solution in a solid–liquid ratio of 1:10 ($w.w^{-1}$). The dilute acid treatment was performed at 130 °C for 15 min in autoclave. The cellulose-rich solid fraction was separated by filtration using qualitative filter paper. The recovered hydrolysate was adjusted to pH 4.0 using NaOH (4.0 M). After neutralization, the hydrolysate was filtered again using qualitative filter paper. The hydrolysate (H-EFB) was used as a source of sugars for fermentation.

Microorganisms, maintenance and spore production medium

Seventeen *Rhizopus* strains were obtained from the collection of the Laboratory of Bioprocess Engineering from the Federal University of Parana. These strains were isolated from previous research work. The identification of these fungi was with a taxonomic key after the detection of typical microscopic structures such as rhizoids, sporangiophore, sporangium and columella [24]. The strains were maintained on PDA agar and grown on 200 g of rice at 30 °C for 15 days, in a 500-mL Erlenmeyer flask to obtain spores. The spores were washed with a sterile tween 80 aqueous solution (0.5%). The spores concentration was adjusted to 1×10^7 $spores.mL^{-1}$.

Screening of fumaric acid-producing Rhizopus strains

The fermentation medium for evaluation of fumaric acid production by different *Rhizopus* species contained: soy peptone (6.0 $g.L^{-1}$), $CaCO_3$ (40.0 $g.L^{-1}$) and a total sugar concentration of 25.0 $g.L^{-1}$ from hydrolyzed (H-EFB). After fermentation, the high fumaric acid-producing strains were selected to evaluated their production using ammonium sulfate (1.5 $g.L^{-1}$) as nitrogen source instead of soy peptone. The pH was kept at 7.2 for both fermentations. The fermentation media were sterilized in an autoclave at 121 °C for 15 min. After sterilization, approximately 1×10^7 spores.mL^{-1} were inoculated and incubated in an orbital incubator at 30 °C, 120 rpm for 96 h.

Fumaric acid production on EFB hydrolysate

The EFB hydrolysate was concentrated by evaporation under vacuum at 45 °C until the total sugar concentration reached 45 g.L^{-1}. The hydrolysate was separated in two groups for fermentation. The first fermentation medium was supplemented with soybean peptone (6.0 g.L^{-1}). The second fermentation medium contained ammonium sulfate (1.5 g.L^{-1}) as nitrogen source. For both fermentation media, $CaCO_3$ (40 g.L^{-1}) was added. The pH of the fermentation medium was adjusted to 7.2. The fermentation media were autoclaved at 121 °C for 15 min. After sterilization, approximately 1×10^7 spores.mL^{-1} of the selected Rhizopus strain were incorporated and incubated on an orbital shaker at 30 °C, 120 rpm for 96 h. Each fermentation experiment was evaluated in duplicate.

Analytical methods

The biomass yield was determined using the dry weight (DW) methodology. Briefly, collected samples were centrifuged at 4,000 rpm for 10 min and the precipitate was washed with a HCl solution (4.0 M) to remove calcium carbonate. The collected biomass was air-dried at 80 °C overnight. The composition of the fermentation medium (glucose, xylose, arabinose, formic acid, acetic acid and by-products such as lactic acid and malic acid) was determined by HPLC (1200, Agilent Technologies, USA) using an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, USA) and refractive index detector (Agilent, HP1047A). For the detection of by-products of hydrolysis and fermentation such as fumaric acid, hydroxymethylfurfural (HMF), and Furfural, an ultraviolet detector (Agilent, G1315D) was used. The column was maintained at 65 °C, with a flow rate of 0.6 mL.min^{-1} and a mobile phase consisting of 5.0 mM H_2SO_4.

Results and discussion

Pretreatment of EFB

The chemical composition of the hydrolysate obtained after dilute acid treatment of EFB is shown in Fig. 1. Xylose was the main monosaccharide found in the hydrolysate (19.82 ± 0.3372 g.L^{-1}), followed by arabinose (2.57 ± 0.0874 g.L^{-1}) and glucose (0.27 ± 0.0037 g.L^{-1}). Cellobiose was also found in the hydrolysate (0.66 ± 0.0327 g.L^{-1}). Hydrolysis by-products such as acetic acid (5.21 ± 0.0634 g.L^{-1}), furfural (0.18 ± 0.0060 g.L^{-1}) and hydroxymethylfurfural (0.01 ± 0.0002 g.L^{-1}) were also detected.

The dilute acid treatment using sulfuric acid was chosen because it has low corrosivity, in relation to membrane and cell wall complexes [25]. The sugar releasing mechanism in this process occurs when sulfuric acid breaks the glycosidic bonds of the hemicellulose chains and the ether bonds of lignin, mainly releasing pentoses. Acetic acid can be formed as a by-product from the action of the acid with the acetylated residues of hemicellulose [26]. Acid hydrolysis used in conjunction with heat treatment enhances the sugars release. In the present work, a hemicellulose release yield of 90% was achieved after dilute acid treatment, where xylose was

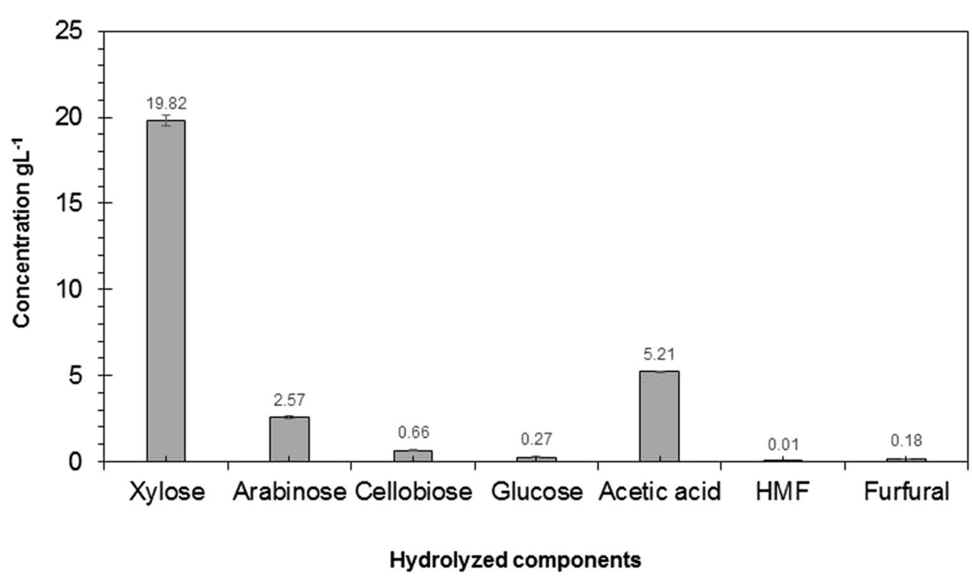

Fig. 1 Chemical composition of EFB hydrolysate

the monosaccharide found in the highest proportion with a concentration of 19.82 $g.L^{-1}$. These values are close to those reported in others work, obtaining 99% extraction of the hemicellulose fraction [27] This indicates that the H-EFB hydrolysate has great potential as carbon source that could be biotechnologically used for the biosynthesis of metabolites of industrial interest.

A disadvantage of dilute acid treatment is that it transforms pentoses and hexoses in a low proportion into molecules with inhibitory activity to cell growth such as furfural and hydroxymethylfurfural (HMF) [28] In addition to this, lignin has been reported to be partially degraded during pretreatment, releasing molecules that could also influence cell growth. In the characterization of H-EFB hydrolysate, several signals were detected (results not shown) at retention times related to lignin residues.

Activation of Rhizopus strains

The growth and spore production on rice medium was not the same for all Rhizopus strains. *R. oligosporium* 25975 showed a spore count of 1.96×10^7 $spores.mL^{-1}$ and it was the best spore producer, followed by *R. arrhizus* 2582, *R. oligosporium* 3267, *R. oryzae* 28168, *R. oligosporium* 2710, and R. 28169. All of them reached an average spore concentration of 1.59×10^7 $spores.mL^{-1}$. In contrast, *R. formosa* 28422 presented the lowest spore concentration (4.96×10^6 $spores.mL^{-1}$). It should be noted that for fermentation and fumaric acid production, the concentration of spores was adjusted to 1×10^7 $spores.mL^{-1}$.

Irregularity in spore production may be due to environmental and genetic factors. The sporulation process is closely related to certain environmental requirements such as high moist content and poor lighting [29]. Rice is a good fungal growth inducer because it contains approximately 90% starch and 15% protein [30] It also contains phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol [31], vitamins such as thiamine, niacin and riboflavin that act as cofactors that, together with aspartic acid and glutamic acid, complement fungal development. Rice is also used as an excellent mean for fungi sporulating used in biological control [32]. From the industrial point of view, rice represents a cheap medium due to its low cost, replacing synthetic growth media such as cassava agar [33] or dextrose potato agar (PDA) [34]. Besides all the advantages described for rice medium, uniformity in sporulation was not obtained, so this result may be due to intrinsic factors of each fungal strain.

Selection of fumaric acid-producing strains

The concentration of fumaric acid produced by each Rhizopus strain was determined at the end of the fermentation process (Fig. 2). R. 395, *R circicans* 1475 and R. 3271, showed significant difference (ANOVA) in the fumaric acid produced compared to the other Rhizopus species tested ($P_{value} < 0.05$). Furthermore, it was determined that R. 3271, *R. delemar* 34612, *R. oligosporium* 3267, and *R. oryzae* 28168, showed significant difference ($P_{value} < 0.05$) in relation to the other Rhizopus species, according to the Tukey

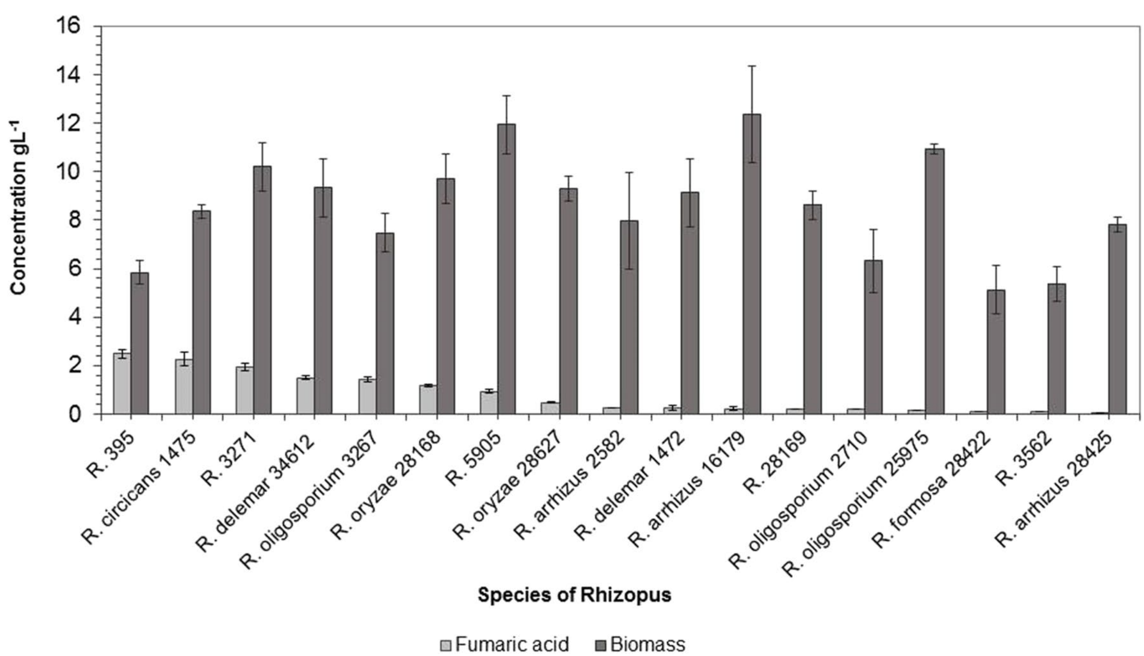

Fig. 2 Rhizopus strain selection by fumaric acid and biomass production. Grey bars represent fumaric acid and dark bars represent biomass

test. It was determined that the form of pellet, mycelium and biofilm were present in the Rhizopus species already mentioned, unlike the others whose predominant form was clamp.

The results indicated that *R. circicans* 1475, R 395, *R. delemar* 34612, R. 3271, *R. oligosporium* 3267, *R. oryzae* 28168 produced fumaric acid in greater proportion than the other 11 isolates ($P_{value} < 0.05$) (Fig. 2). Obviously, this result corresponds to the fact that not all Rhizopus species are fumaric acid-hyperproducers. In fact, a research pointed out that there are certain Rhizopus species with the capacity to produce mainly fumaric and malic acid, unlike others that only produce only L-lactic acid [35]. Some works indicated that *R. oryzae* and *R. arrhizus* were good producers of fumaric acid, using glucose and xylose as carbon source [36–38]. In the present work, the first 6 Rhizopus species were selected in order to evaluate their capacity for producing fumaric acid using the pentoses present in the EFB hydrolysate. The other Rhizopus species were discarded due to their low fumaric acid production.

The six selected fungi were further evaluated in the EFB hydrolysate medium supplemented with two different nitrogen sources (Fig. 3). The fumaric acid concentration (4.65 g/L) obtained using *R. circicans* 1475 and soy peptone as nitrogen source was remarkable ($p < 0.05$). *R.* 395, *R. delemar* 34,612 and *R.* 3271 presented similar fumaric acid concentrations (3.23, 2.60 and 2.54 g/L) respectively. *R. circicans* 1475, R. 395 and R 3271 were noted for their high concentrations of fumaric acid using ammonium sulfate (Fig. 3A).

Soy peptone and ammonium sulfate have been described as inducers of the form that fungal biomass acquires in cell growth processes [39] From Fig. 3B, it was possible to observe that the pellet form was characteristic when fungi were grown in soy peptone as nitrogen source, obtaining a diameter varying between 1 to 3 mm in length. On the other hand, when ammonium sulfate was used as nitrogen source, fungi acquired the form of mycelia (0.08 – 3 mm in length). *R. delemar* 34612 and *R. oryzae* 28168 were recognized as clams and biofilm respectively. These results agreed with those presented in other work [40]. The importance of obtaining fungal biomass in the form of pellets or mycelia relies on the fact that compact and small configurations increase the contact surface for greater gas exchange and higher sugar consumption [41]. In this work, there was no clear evidence that any specific fungal form enhanced the

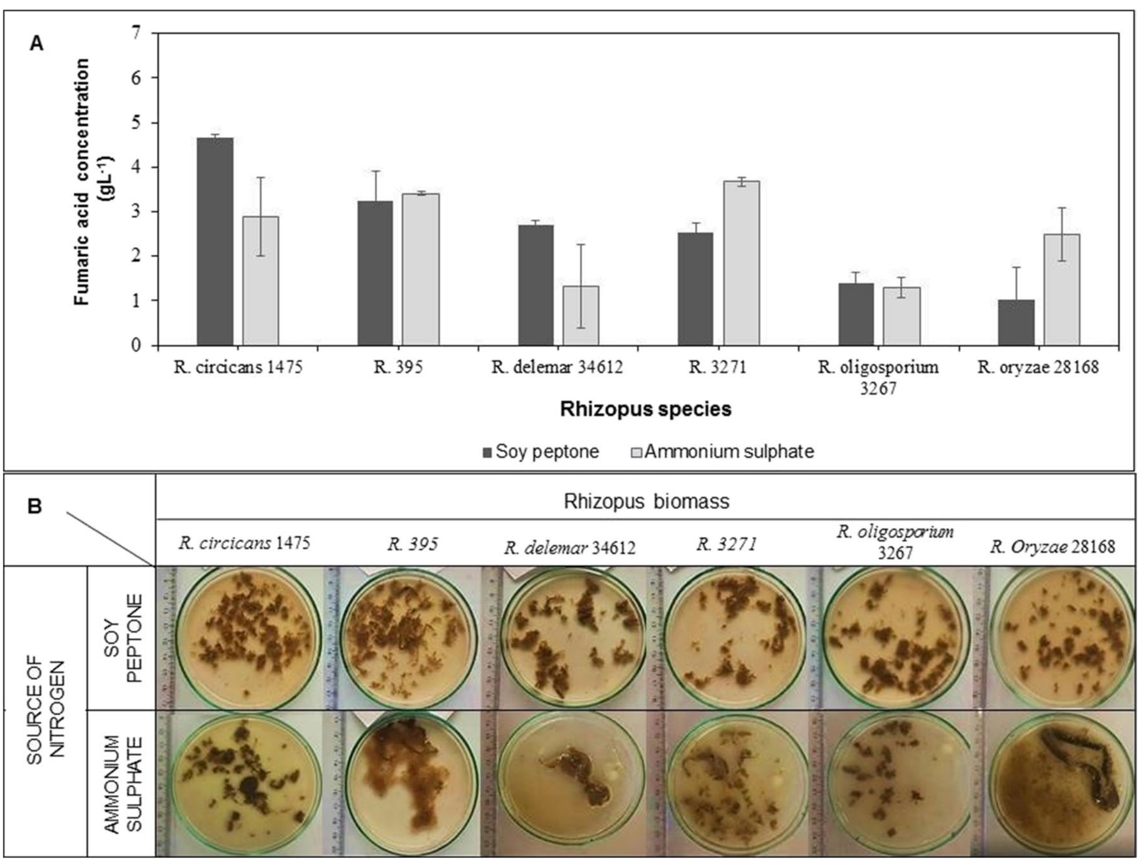

Fig. 3 Fumaric acid production using EFB hydrolysate with soy peptone and ammonium sulfate as nitrogen sources. **A** Fumaric acid production, Grey bar: soy peptone and dark bars: ammonium sulfate. **B** morphology at the end of the fermentation

fumaric acid production. *R. circicans* 1475, R. 395 and R. 3271 were selected to perform the fermentation experiments in larger scale and evaluating other nitrogen sources.

Fumaric acid production on EFB hydrolysate

Table 1 shows the final sugar concentration, biomass and fumaric acid produced by the three selected fungi (*R. circicans* 1475, *Rhizopus* 392 and *Rhizopus* 3271) using two different nitrogen sources (soy peptone and ammonium sulfate) and the pentose-rich hydrolysate (45 g.L^{-1}). It was observed that R. 3271 and *R. circicans* 1475, using soy peptone and ammonium sulfate, were the highest fumaric acid producers.

Several studies reveal the capacity that members of the Rhizopus genus have for the production of fumaric acid with glucose and xylose, indicating productions of up to 45 g. L^{-1} and yields of up to 73% [42, 43]. Other experiences that try to take advantage of agro-industrial residues as a carbon source use treatments to detoxify their hydrolysates, reducing microbial growth inhibitors [44]. However, the challenge remains to use hydrolyzed acids from agroindustrial residues that, in addition to presenting sugars (a mixture of xylose and glucose), also present inhibitors of microbial growth (furfural and HMF). Fermentations carried out with hydrolyzed acids rich in pentose indicate that 5.8 g.L $^{-1}$ of fumaric acid are produced. Therefore, our results stand out in relation to others [45], for producing fumaric acid using hydrolyzed acids. of EFB, rich in xylose and without any detoxification process, obtained results similar to those without optimization processes.

Regarding the remaining sugar concentration, the fungi exposed to ammonium sulfate exhibited higher sugar consumption (41–47%) than those fungi exposed to soy peptone (24–34%). Thus, the nitrogen source resulted to be an important factor in the production of fumaric acid and sugar consumption. Nevertheless, the diameter of the fungal pellets showed no significant difference between the nitrogen sources employed. *R. circicans* 1475 exhibited the highest fumaric acid yield using soy peptone as nitrogen source.

The fungi exposed to EFB hydrolysate may have experienced different inhibitory effects such as those derived from furfural. In fact, the concentration of furfural in the concentrated fermentation medium was 0.5 g.L^{-1}. It has been reported that concentrations greater than 0.6 g.L $^{-1}$ can cause inhibition of *Rhizopus*, as it can affect enzymatic activity, interfering with RNA synthesis and causing DNA damage [46, 47]. Another inhibitor of microbial growth is acetic acid, whose concentration in the fermentation medium was 10.42 g.L^{-1}. Some fungi such as *Aspergillus* have been reported to present little biomass generation when exposed to a concentration of 5.0 g.L^{-1} of acetic acid, although *R. oryzae* was not inhibited at this concentration [48]. It should be noted that this research has not evaluated alternatives for the elimination of these inhibitors found in the fermentation medium since it would increase the processing costs in the valorization of the agro-industrial waste.

An interesting work to analyze is the one developed by [49]. It shows the production of fumaric acid from EFB sugars. Initially they obtained a productivity of 5.3 ± 0.030 g. L^{-1} (similar to our production) and after an optimization process it increased by 8.32 times, reaching 44 g.L^{-1}. In the pretreatment of their substrate, they destroyed the EFB fibers with steam explosion, then performed delignification with 15% NaOH, subsequently they made washes to eliminate residues and finally performed enzymatic hydrolysis. This pretreatment is interesting because it reduces the concentration of metabolic inhibitors such as furfural, hydroxymethyl furfural or others present in hydrolysates like ours. Despite using this purified hydrolyzate compared to our work (hydrolyzed with metabolic inhibitors) we had similar fumaric acid productions (5.65 g.L^{-1} and 5.3 g.L^{-1}); However, in our opinion the big difference lies in its optimization process, which consisted of using an Air Lift bioreactor, with an aeration rate of 1.5 VVM and a high C/N range. From a metabolic point of view, this favors the fumaric acid production pathway.

It is important to note that even that the Rhizopus strains were able to consume the pentose-rich hydrolysate from EFB for the production of fumaric acid, some research is

Table 1 Fumaric acid production by Rhizopus species using EFB hydrolysate and two different nitrogen sources

Fungi	Soy peptone					Ammonium sulfate				
	S. C. (g.L^{-1})	F. P (g.L^{-1})	B. P (g.L^{-1})	Y(p/s)	Y(x/s)	S. C. (g.L^{-1})	F. P (g.L^{-1})	B. P (g.L^{-1})	Y(p/s)	Y(x/s)
R. circicans										
1475	11.20 ± 0.33	5.65 ± 0.27	6.80 ± 0.6	0.51	0.61	18.48 ± 0.52	3.98 ± 0.62	8.18 ± 0.30	0.22	0.44
R 392	14.78 ± 2.15	4.61 ± 0.15	9.94 ± 0.55	0.31	0.67	18.75 ± 2.63	3.75 ± 0.60	9.05 ± 1.73	0.20	0.48
R 3271	15.31 ± 2.09	5.25 ± 0.92	9.03 ± 0.61	0.34	0.59	21.59 ± 1.72	4.48 ± 0.15	14.18 ± 1.87	0.21	0.66

S.C: Sugar concentration

F.P: Fumaric acid production

B. P: Biomass production

still encouraged in order to improve the production. Some authors have carried out studies at the metabolic engineering level to optimize the bioprocess. One strategy is to add small amounts of certain molecules such as nicotinic acid (source of NADH), vitamin C, sodium citrate, and amino acids such as serine and proline, the latter to regulate the metabolic flow from malate to fumarate, and increasing fumaric acid production from 6.5 g.L^{-1} to 20.2 g.L^{-1} [45]. Another way to increase the fumaric acid production is the overexpression of genes that express the pyruvate carboxylase and phosphoenolpyruvate decarboxylase enzymes, increasing the flux of carbon fixation for the formation of oxaloacetate, malate and fumarate [50].

This research was intended to valorize oil palm empty fruit bunches by producing fumaric acid using the pentose-rich hydrolysates from EFB in a single fermentation process. This approach reduces costs and production times. However, despite of having obtained similar results to other investigations, it is important to consider the idea of optimizing the Rhizopus fungal biomass separately. In this way, a suitable optimization process can be carried out.

Finally, our research offers a new alternative to the problem of lignocellulosic residues generated in the palm oil industry, which causes environmental damage such as the proliferation of insects or alteration of the pH in water and soil. The valorization of the hemicellulosic fraction of EFB for the production of organic acids makes this biomass attractive to reduce environmental pollution and to produce fumaric acid in an environmentally friendly way. Undoubtedly, taking advantage of this waste, Brazil could enhance its participation in the international market, consolidating a biotechnological platform based on these acids. Nevertheless, more investigation is encouraged to improving the fermentation processes of Rhizopus and the hydrolysates of EFB.

Conclusions

In this study, seventeen Rhizopus species were evaluated to make use of the pentoses released from the hemicellulosic fraction of EFB, producing fumaric acid and valorizing an agro-industrial residue. The effect of the initial sugar concentration of 45 g.L^{-1} in the culture media on the fumaric acid production was evaluated. 5.65 ± 0.27 g.L^{-1} of fumaric acid was obtained by *R. circicans* 1475 and 5.25 ± 0.92 g.L^{-1} by *Rhizopus* 3271, with initial total sugar concentration of 45 g.L^{-1}, using soybean peptone as a nitrogen source. *R. circicans* 1475 and *Rhizopus* 3271 were the best fumaric acid producers found. It is important to highlight that to increase the fumaric acid production, more research is encouraged to unravel the metabolic bottlenecks that hinder its biosynthesis. In addition, the overexpression of genes related to fumaric acid formation should be considered. Finally, other alternatives to eliminate cell growth inhibitors must be explored since they greatly affect the development of the microorganisms. These research approaches help to improve the fumaric acid production, allowing in the near future the proper use of agro-industrial residues in a sustainable way.

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Declarations

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

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