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Ethanol production by co-culture of *Zymomonas mobilis* and *Pachysolen tannophilus* using banana peels hydrolysate as substrate

Juliana Ferreira[1*], Vidiany Aparecida Queiroz Santos[2] and Crispin Humberto Garcia Cruz[1]

*[1]Departamento de Engenharia e Tecnologia de Alimentos, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rua Cristóvão Colombo, 2265, 15054-000, Jardim Nazareth, São José do Rio Preto, São Paulo, Brazil. [2]Departamento de Química, Universidade Tecnológica Federal do Paraná, Pato Branco, Paraná, Brazil. *Author for correspondence. E-mail: ju_ferreirams@hotmail.com*

ABSTRACT. Ethanol production by *Zymomonas mobilis* CCT 4494 and *Pachysolen tannophilus* CCT 1891 co-cultures and individual fermentation from each microorganism are evaluated with hydrolysate of banana peels. Banana peels hydrolysate was obtained by sulfuric acid pretreatment followed by enzymatic saccharification, carried out on solid fraction and used as substrate by individual and co-cultures of *Z. mobilis* and *P. tannophilus* fermentation. Effect of pH and total solids concentration of banana peels hydrolysate on ethanol production was also evaluated. Pretreatment followed by enzymatic hydrolysis was efficient and produced a medium with total sugar concentration of 72.80 g L^{-1} and sugar reducing concentration of 52.90 g L^{-1}. *Z. mobilis* and *P. tannophilus* co-culture were more efficient than individual strains. *P. tannophilus* inoculated after *Z. mobilis* substantially improved ethanol production. The highest ethanol production using banana peels hydrolysate by microbial co-fermentation was 11.32 g L^{-1}, at a productivity of 0.60 g L^{-1} h^{-1}, reaching 77.59% of the maximum theoretical yields.

Keywords: bioethanol; co-fermentation; bacteria; yeast, hydrolysate.

Produção de etanol por cofermentação de *Zymomonas mobilis* e *Pachysolen tannophilus* utilizando hidrolisado de cascas de banana como substrato

RESUMO. O objetivo desse estudo foi avaliar a produção de etanol por cocultura de *Zymomonas mobilis* CCT 4494 e *Pachysolen tannophilus* CCT 1891 e fermentação individual de cada microrganismo usando hidrolisado de casca de banana. As cascas de banana foram pré-tratadas com ácido sulfúrico, seguido de hidrólise enzimática, a qual foi realizada na fração sólida das cascas de banana. Após esse processo, foram avaliados o efeito da concentração de sólidos totais e do pH inicial do meio à base do hidrolisado nos ensaios fermentativos com culturas individuais e coculturas de *Z. mobilis* e *P. tannophilus*. O pré-tratamento seguido de hidrólise enzimática foi eficiente, resultando em um meio com concentração de açúcares totais de 72,80 g L^{-1} e de açúcares redutores de 52,90 g L^{-1}. A cocultura de *Z. mobilis* e *P. tannophilus* apresentou maior eficiência do que as culturas individuais. A inoculação de *P. tannophilus* após a *Z. mobilis* resultou em aumento significativo na produção de etanol. Nos ensaios conduzidos utilizando cofermentação de *P. tannophilus* e *Z. mobilis* em meio à base de hidrolisado de cascas de banana, a maior produção de etanol observada foi de 11,32 g L^{-1}, com produtividade de 0,60 g L^{-1} h^{-1}, alcançando 77,59% de rendimento teórico máximo.

Palavras-chave: bioetanol; cofermentação; bactéria; levedura; hidrolisado.

Introduction

Oscillating oil prices, environmental problems due to conventional fuels and increasing demand for fossil fuel have urged the scientific community worldwide to intensify research on alternative and renewable energy sources (Gabhane, Prince Willian, Vaidya, Mahapatra, & Chakrabarti, 2011). Lignocellulosic materials are promising sources of renewable raw material for various biotechnological processes due to their low economic value and high availability (Silva, Carneiro, & Roberto, 2013). In fact, the biomass may be utilized to produce ethanol, a promising alternative energy source for limited crude oil (Sathesh-Prabu & Murugesan, 2011).

Driven by rising global demand, high prices and technological advances, agricultural production in Brazil has steadfastly increased over the last two decades, taking advantage of the country´s immense agricultural resources available (United States Department of Agriculture [USDA], 2016). Banana production and consumption, globally and locally, stand out among agricultural products. Banana crop ranks second place in fruit volume produced in Brazil, following by orange production. According to the Brazilian Institute of Geography and Statistics

(IBGE), the banana production, cultivated in 478 thousand hectares at an average yield of 14.5 kg ha^{-1}, reached approximately 7 million tons in 2014 (Instituto Brasileiro de Geografia e Estatística [IBGE], 2016).

A small portion of total banana production in Brazil is subject to some industrialization process, totaling only 2.8% (Moraes, 2011), that is, most of it is consumed *in natura* and has a short shelf life. Besides, banana peels features 40% of its weight, there is an annual industrial waste of about 86 tons of banana peels. An alternative for reusing this raw material, commercially worthless, is its employment as substrate for biotechnological purposes, specifically for ethanol fermentation, especially due to the proximate compositional. Oberoi, Sandhu, and Vadlani (2012) suggests that besides sugars, banana peels are rich in cellulose (12%), hemicellulose (10%), pectin (15.9%), lignin (2.9%) and ash (9.8%).

These factors associated to its low cost and high availability turn banana peels an interesting residue that could be used as raw material in different processes. Banana peels can be used as effective adsorbent for metal removal from aqueous solution and industrial waste water (Ali, 2017), and as base material for pectin extraction (Swamy & Muthukumarappan, 2017). Besides that, banana peels are a profitable source of bioactive compounds (Rebello et al., 2014), and also can be used as substrate to ethanol production by microbial fermentation.

During the last thirty years, ethanol-production using bacterium *Z. mobilis* has been in the biotechnology limelight. *Z. mobilis* metabolizes sugar by the Entner–Doudoroff (ED) pathway, producing less ATP and biomass and more ethanol. Other carbon sources are also used in ethanol production by *Z. mobilis*, resulting in higher ethanol yield than that by native ethanol fermenting yeast *Saccharomyces cerevisiae* (Bai, Anderson, & Moo-Young, 2008). In literature, many studies using *Z. mobilis* to ethanol production by lignocellulosic feedstocks fermentation can be found, but this bacterium is used in association with another microorganism, because it is not able to metabolize pentoses, as xylose. Therefore, there are no any microorganisms which are naturally capable of efficiently converting lignocellulosic feedstock sugars. Moreover, an efficient conversion of glucose and xylose is a requisite for the profitable process of bioethanol production from lignocellulosic substrates. Among several approaches available for such conversion are extant, co-culture is a simple process, employing two different organisms for the fermentation the fermentation of two or more sugars present in fermentation medium (Fu, Peiris, Markham, & Bavor, 2009).

Pichia stipitis, *Candida shehatae* and *Pachysolen tannophilus* are natural yeasts that ferment xylose and produce ethanol (Sánchez, Bravo, Castro, Moya, & Camacho, 2002; Agbogbo, Coward-Kelly, Torry-Smith, & Wenger, 2006; Fu & Peiris, 2008). Among these strains, *Pachysolen tannophilus* is a promising yeast in biochemical processes leading to bioethanol synthesis from pretreated lignocellulosic materials due its ability to convert hexoses and pentoses (Castro, Ginovart, & Gras, 2010). Current study analyzes the hydrolysis process of banana peels and bioconversion of the hydrolysate into ethanol by *Z. mobilis* CCT 4494 and *P. tannophilus* CCT 1891 co-cultures and individual fermentation from each microorganism.

Material and methods

Collection and processing of banana waste

Ripe banana peels (*Musa cavendischii)* were used as raw material, acquired from the university restaurant of the *Instituto de Biociências, Letras e Ciências Exatas* (IBILCE/UNESP), in São José do Rio Preto, São Paulo, Brazil, at 20º49'10.99" S and 49º22'45.98" W.

Banana peels were cut manually into pieces smaller than 3 cm and dried in solar light for approximately 24h, until they became stiff and brittle. Banana peels presented moisture of 12.4% (w w^{-1}). The dried husks were then ground in a knife mill producing a powdered biomass which was sieved (series - 14) obtaining a particle size of less than 1.41 mm. Then, powdered biomass was packed and stored in glass vials at room temperature.

Acid and enzymatic hydrolysis

Acid hydrolysis was carried out according to procedure described by Monsalve Gil, Perez, and Colorado (2006), with modifications. Powdered banana peels (10 g) were placed in a 250 mL Erlenmeyer flask and suspended in 50 mL of 5% H_2SO_4 (v v^{-1}). Hydrolysis was performed in autoclave at 121°C, with 15 and 30 min. pretreatment time for all experiments. After pretreatment, the digested material was cooled and neutralized by 50% NaOH (w v^{-1}). The material was filtered with filter paper and used to determine total and reducing sugars content.

The solid fraction from the acid hydrolysis step was used as substrate for enzymatic hydrolysis. Enzymatic hydrolysis experiments were performed in a 0.1 mol L^{-1} sodium citrate buffer (pH 5.5), in 250 mL Erlenmeyer flasks with a rotary shaker at 100 rpm and 50°C. The final

workload was 100 mL for all assays. Enzymatic mix used was composed by Cellulase complex (NS22086), Xylanase (NS22083), β-glucosidase (NS22118) and Enzyme complex (NS22119) and were obtained from Novozymes (Novozymes Latin America Ltda., Araucaria, Brazil). Enzymes dosages followed manufacturer's instructions. Samples were taken after 24 h, filtered through filter paper and an aliquot was analyzed for total and reducing sugars concentrations, as described below.

Hydrolyzed detoxification

Hydrolyzed samples were mixed in activated charcoal (Merck) (40:1 w w^{-1}; sample: charcoal) and incubated at 30°C and 200 rpm during 1h. Samples were then centrifuged at 3000 g for 20 min. and filtered with filter paper to remove the charcoal.

Microorganisms, culture medium and preparation of inoculum

Z. mobilis CCT 4494 and P. tannophilus CCT 1891 were obtained in the André Tosello Foundation (Campinas, Brazil). Z. mobilis was cultivated and maintained in medium (g L-1) composed of glucose (20), peptone (10), yeast extract (10) and pH adjusted to 5.5 using HCl or NaOH (0.1 mol L-1). Z. mobilis was reactivated monthly in the maintenance medium and incubated at 30°C for 24h. P. tannophilus CCT 1891 was cultivated and maintained in medium (g L-1) composed of glucose (10), peptone (5), yeast extract (3), malt extract (3), agar (20) and pH adjusted to 5.5 (maintenance medium). Yeast was reactivated monthly in maintenance medium and incubated at 28 °C for 24h. Both microorganisms were stored at 4°C in their respective maintenance medium.

For inoculums preparation, strains were previously reactivated in maintenance medium and incubated in a bacteriological incubator during 24h, at 30 and 28°C, by *Z. mobilis* and *P. tannophilus* respectively. Inoculums were prepared separately for each strain in 250 mL Erlenmeyer flasks, containing 50 mL of maintenance medium and the incubation conditions was similar to described for strains reactivation. Inoculum was centrifuged at 6941 g 15 $min.^{-1}$; and the supernatant was discarded. Then, the cell pellet was resuspended in sterile distilled water to obtain a cell suspension standardized by turbidimetry at optical density (0.6) in 570 nm using a spectrophotometer (Biochrom, Libra S22, Cambridge, UK).

Ethanol production

Ethanol was produced with basal medium composed of yeast extract (5 g L^{-1}); $MgSO_4.7H_2O$ (1 g L^{-1}); $(NH_4)_2SO_4$ (1 g L^{-1}) and KH_2PO_4 (1 g L^{-1}) (Rodríguez & Callieri, 1986), and banana peels hydrolysate was used as carbon source. Total Solids (TS) of banana peels hydrolysate were adjusted at different concentrations (5, 10 and 15%) using a refractometer (Biobrix, São Paulo, Brazil). Banana peels hydrolysate was sterilized separately from salts. First, fermentation was carried out using each microorganism separately. *Z. mobilis* was cultivated at 30°C during 7h and *P. tannophilus* during 19h at 30°C. Co-fermentation assays using *Z. mobilis* and *P. tannophilus* was carried out by two methods.

In first co-culture (co-fermentation 1) assay, the two microorganisms were inoculated at the same time and incubated at 30°C during 19h. Fermentations were carried out in 250 mL Erlenmeyer flasks, containing 50 mL of fermentation medium, incubated in shaker, at 30°C and 100 rpm. In second co-culture (co-fermentation 2) experiment, only *Z. mobilis* was inoculated and after fermentation during 7h at 30°C, the fermentation medium was centrifuged, bacterial cells were removed, and then, the yeast *P. tannophilus* was added and fermentation was conducted at 30°C during 19h. Effect of initial pH (4.5, 5.0 and 5.5) and total solids concentration (TS) (5, 10 and 15%, which correspond to total sugar concentration of 16.8, 29.8 and 57.2 g L^{-1}, respectively) of banana peels hydrolysate on the ethanol production were assessed. Fermentation process was conducted in triplicate.

Analytical methods

Moisture content in the dried banana peels were analyzed by AOAC method (Association of Official Analytical Chemists [AOAC], 2000). Phenolic compounds in banana peels hydrolysates were evaluated before and after detoxification. Total phenolics content were determined with modified Folin-Ciocalteu method described by Chaovanalikit and Wrolstad (2004). Final pH was determined directly in fermentation medium by potentiometry (Digmed, DM20, São Paulo, Brazil). Cell growth was determined by turbidimetry at 570 nm, relating the biomass content with dried biomass calibration curve. Total sugar concentration was estimated by the phenol-sulfuric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Reducing sugars were estimated by Somogyi (1952) and Nelson

(1944) method and total solid soluble content was determined with a refractometer (Biobrix, São Paulo, Brazil). Ethanol was evaluated by gas chromatography using GC Focus-Thermo Scientific, TR-wax column 30x0.053, IDx1μm. Oven temperature was maintained at 100°C, with injector and detector temperatures at 230 and 270°C, respectively. Nitrogen was used as carrier gas whilst productivity (g L^{-1} h^{-1}) and substrate consumed (g L^{-1}) were calculated by Equations 1 and 2, respectively.

$$P = \frac{(P_f - P_0)}{(T_f - T_0)} \quad (1)$$

$$S = (S_f - S_0) \quad (2)$$

where P_0 = initial product mass (g L^{-1}); P_f = final product mass (g L^{-1}); S_0 = initial substrate concentration (g L^{-1}); S_f = final substrate concentration (g L^{-1}); t_f = final time (h); t_0 = initial time (h).

Results and discussion

Acid pretreatment

Figure 1 shows the influence of time on the effectiveness of pretreatment. Results indicated that practically there was no difference in sugar concentration as pretreatment time increased. There was a slight reduction in total sugar concentration from 46.7 g L^{-1} in 15 min. to 44.8 g L^{-1} in 30 min. Increase in exposition time of lignocellulosic materials at high temperatures may lead to degradation of sugars. This behavior was described by (Phitsuwan, Sakka, & Ratanakhanokchai, 2013). These authors highlight that high temperatures may promotes the sugar monomers (hexose) degradation, e.g. glucose, to 5-hydroxymethyl furfural (HMF), whereas xylose is degraded to furfural. Based on these results, time for further pretreatment experiments was fixed at 15 min. in current analysis.

Due to enzymatic hydrolysis, there was a 55% increase in total sugar concentration compared with pretreatment (46.68 g L^{-1}), whilst pretreated broth by acid and enzymatic hydrolysis presented 72.80 g L^{-1} of total sugar concentration. Reducing sugar also increased from 29.55 to 52.90 g L^{-1} after enzymatic hydrolysis. Reducing sugar rates were close to those reported by Oberoi et al. (2012) who hydrolyzed dried ground banana peels with a combination of cellulolytic and pectinolytic enzymes, and verified 48 g L^{-1} of reducing sugar concentrations. According Alvira, Tomás-Pejó, Ballesteros, and Negro (2010) acid pretreatment is performed to solubilize the hemicellulosic fraction of the biomass and to increase the enzyme accessibility improving digestibility of cellulose.

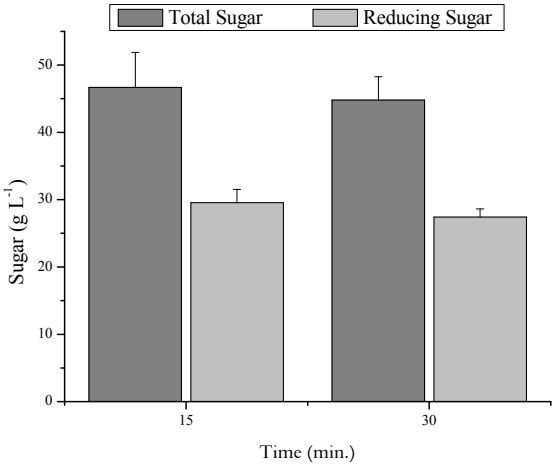

Figure 1. Influence of pretreatment time on total and reducing sugars content from banana peels submitted to acid pretreatment.

Hydrolysate detoxification

Hydrolysate submitted to acid and enzymatic hydrolysis pretreatments was detoxicated with activated charcoal to minimize inhibitory compounds. This process is necessary because several inhibitory compounds to microbial metabolism were also liberated and/or formed during the pretreatment (Canilha, Carvalho, Felipe, Almeida e Silva & Giulietti, 2010) and may slow growth kinetics and limited yields during the fermentative process (Todhanakasem, Sangsutthiseree, Areerat, Young, & Thanonkeo, 2014).

Table 1 presents sugars and phenolic compounds in non-detoxified and detoxified hydrolysates. There was no significant interference ($p < 0.05$) on total sugar reduction after detoxication. This behavior is very important for glucose utilization during ethanol biosynthesis. On the other hand, concentration of phenolic compounds decreased by ≅ 80% after treatment with active charcoal ($p < 0.05$).

Table 1. Composition of non-detoxified and detoxified hydrolysates.

Hydrolysate	Total Sugar (g L^{-1})	Total Phenolic (g L^{-1})
Non-detoxified	72.80 ± 3.77[a]	1.01 ± 0.39[a]
Detoxified	70.90 ± 2.20[a]	0.17 ± 0.02[b]

[a,b]... (column) – means followed by the same small letters do not differ by Tukey´s test ($p < 0.05$).

Toxic compounds in the fermentation medium, such as the phenolic compounds in current study, may be a stress factor for microorganisms, causing reduction in sugar utilization and product formation

(Todhanakasem et al., 2014). Canilha et al. (2010) and Chandel, Kapoor, Singh, and Kuhad (2007) observed a respective 1.2 g L^{-1} and 3.9 g L^{-1} increase in ethanol production using detoxified hydrolysates. Silva et al. (2013) verified that ethanol volumetric productivity by *P. stipitis* doubled when compared to non-treated hydrolysate.

Ethanol production by *Z. mobilis* using banana peels hydrolysate as substrate

Banana peels hydrolysate at three different concentrations of total solids (5, 10 and 15%) at initial pH (4.5, 5.0 and 5.5) was evaluated by ethanol production using *Z. mobilis* as fermenting microorganism. Figure 2 and Table 2 reveal higher ethanol production and productivity (2.90 g L^{-1}; 0.42 g L^{-1} h^{-1}) obtained at initial pH 5.0 and using 5% hydrolysate, moreover it was obtained a high yield compared with the maximum theoretical yield (75.12%). Ethanol production less than 1 g L^{-1} occurred in other conditions evaluated. Similar results were reported by Monsalve Gil et al. (2006) who evaluated acid hydrolysis of banana peels by ethanol production using *Z. mobilis* and verified ethanol production less than 0.1 g L^{-1}.

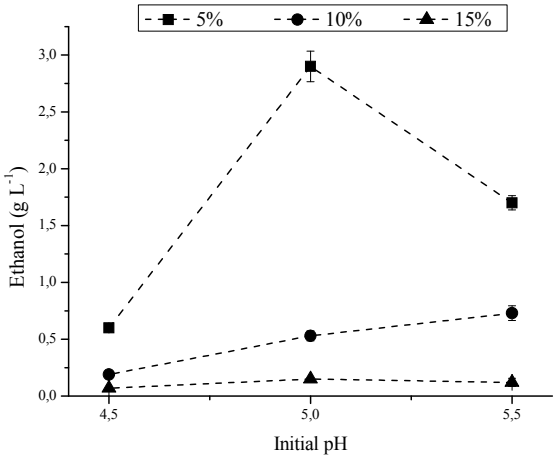

Figure 2. Effect of substrate concentration and initial pH of banana peels medium in ethanol production by *Z. mobilis*.

Table 2 reveals that *Z. mobilis* presented low biomass production (0.10 - 0.27 g L^{-1}) in all conditions tested. Higher biomass production did not promote higher ethanol biosynthesis. According to Table 2, a higher rate (0.27 g L^{-1}) was observed when 15% of banana peels hydrolysate and initial pH 5 and 5.5 were employed. In addition, Table 2 shows substrate concentrations consumed in every assay. In order to supply enough energy for growth, *Z. mobilis* bacterium catabolizes the substrate with high specific rates, resulting in low biomass yields (Toma et al., 2003).

Table 2. Cell biomass, substrate consumed and ethanol productivity observed after fermentation of banana peels medium by *Z. mobilis*.

Substrate concentrations	Cell biomass (g L^{-1})	Substrate Consumed (g L^{-1})	Productivity (g L^{-1} h^{-1})
pH 4.5			
5%	0.13 ± 0.04	6.13 ± 1.31	0.09
10%	0.18 ± 0.03	4.22 ± 2.87	0.03
15%	0.16 ± 0.04	17.04 ± 1.10	0.01
pH 5.0			
5%	0.10 ± 0.03	7.58 ± 1.65	0.42
10%	0.12 ± 0.02	7.12 ± 0.56	0.08
15%	0.27 ± 0.04	20.47 ± 0.19	0.02
pH 5.5			
5%	0.16 ± 0.02	8.29 ± 0.48	0.24
10%	0.20 ± 0.03	5.64 ± 1.94	0.10
15%	0.27 ± 0.01	21.37 ± 2.21	0.02

Ethanol production using banana peels hydrolysate broth by *P. tannophilus*

Figure 3 and Table 3 show that P. tannophilus presented a completely different behavior from that shown by Z. mobilis. The yeast showed high ethanol production and productivity (4.01 g L^{-1}; 0.21 g L^{-1} h^{-1}) with 5% banana peels medium and initial pH 4.5 and it was obtained a yield of 62,56% compared with the maximum theoretical yield. Ethanol production was better in lower substrate concentration evaluated and consequently, there was reduction in substrate concentrations of 10 and 15%, according showed in Figure 3.

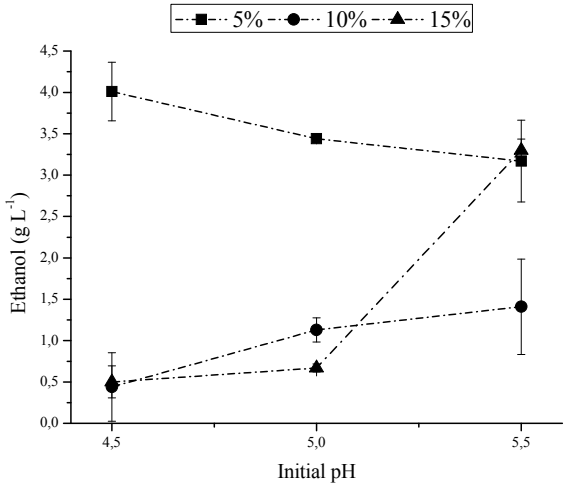

Figure 3. Effect of substrate concentration and initial pH of banana peels medium in ethanol production by *P. tannophilus*.

High biomass rate (3.80 g L^{-1}) was observed in fermentations using banana peels medium 5% and initial pH 5.5 as Table 3 show. The reduced

content of inhibitor compounds present at this low hydrolysate concentration may be responsible by higher cellular biomass concentrations observed in 5% of banana peels hydrolysate.

Table 3. Cell biomass, substrate consumed and ethanol productivity observed after fermentation of banana peels medium by *P. tannophilus*.

Substrate concentrations	Cell biomass ($g\ L^{-1}$)	Substrate consumed ($g\ L^{-1}$)	Productivity ($g\ L^{-1}\ h^{-1}$)
pH 4.5			
5%	3.18 ± 0.24	12.58 ± 0.94	0.21
10%	1.03 ± 0.13	12.81 ± 0.88	0.02
15%	0.68 ± 0.10	24.72 ± 1.21	0.03
pH 5.0			
5%	3.30 ± 0.38	14.04 ± 0.73	0.18
10%	2.00 ± 0.27	7.73 ± 1.54	0.07
15%	2.14 ± 0.25	25.93 ± 2.71	0.04
pH 5.5			
5%	3.80 ± 0.23	14.58 ± 0.37	0.17
10%	2.37 ± 0.39	10.03 ± 1.06	0.07
15%	1.03 ± 0.08	27.83 ± 1.60	0.17

Ethanol production by *Z. mobilis* and *P. tannophilus* using banana peels hydrolysate medium (co-fermentation 1)

In co-fermentation 1, both microorganisms were inoculated at the same time and the effect of different substrate concentrations and initial pH was assessed. Figure 4 describes the behavior of co-culture in banana peels medium by ethanol production, with high rate 4.92 $g\ L^{-1}$ and productivity 0.26 $g\ L^{-1}\ h^{-1}$ when substrate 10% and initial pH 5.5 were employed.

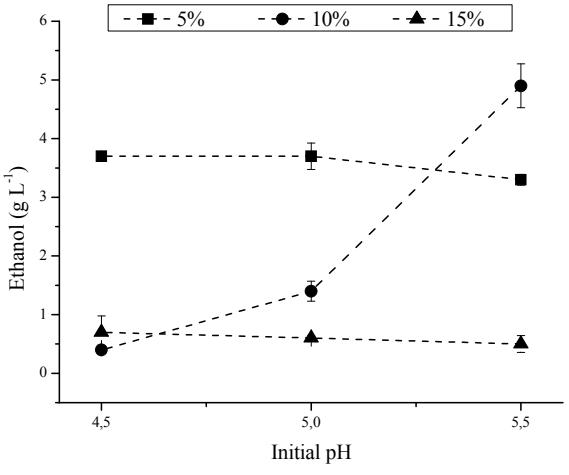

Figure 4. Effect of substrate concentration and initial pH of banana peels hydrolysate medium in ethanol production by co-fermentation of *Z. mobilis* and *P. tannophilus* (co-fermentation 1).

Similar to fermentations realized with individual microorganism (Tables 2 and 3), biomass growth in the co-culture was higher in substrate 5% and initial pH 5.5 (Table 4). On the other hand, substrate consumption increased when a greater substrate concentration was employed (15%), although it was not used by ethanol or biomass production.

Co-culture had better results when compared to experiment with *Z. mobilis* alone, showing increase from 2.90 to 4.92 $g\ L^{-1}$ in ethanol production; in the case of *P. tannophilus*, simultaneous fermentation increased slightly ethanol biosynthesis from 4.01 to 4.92 $g\ L^{-1}$.

Table 4. Cell biomass, substrate consumed and ethanol productivity observed after fermentation of banana peels medium by co-culture of *Z. mobilis* and *P. tannophilus* (co-fermentation 1).

Substrate concentrations	Cell biomass* ($g\ L^{-1}$)	Substrate Consumed ($g\ L^{-1}$)	Productivity ($g\ L^{-1}\ h^{-1}$)
pH 4.5			
5%	1.01 ± 0.03	11.88 ± 0.51	0.20
10%	0.22 ± 0.04	2.98 ± 2.55	0.02
15%	0.63 ± 0.41	30.10 ± 0.45	0.04
pH 5.0			
5%	1.60 ± 0.03	12.91 ± 0.02	0.20
10%	0.31 ± 0.02	3.80 ± 1.99	0.07
15%	0.12 ± 0.12	31.69 ± 2.50	0.03
pH 5.5			
5%	1.92 ± 0.04	12.74 ± 0.26	0.17
10%	0.92 ± 0.04	14.02 ± 0.83	0.26
15%	0.21 ± 0.13	31.70 ± 2.51	0.02

*Biomass quantification refers to the mixed culture of two strains, *Z. mobilis* and *P. tannophilus*, used in this fermentation.

These low results obtained in the co-culture experiment with bacteria and yeast inoculated simultaneously on fermentation medium may have been caused by competition between strains. Similar observation has been made for *Z. mobilis* in the co-culture with *Pichia stipitis* (Fu et al., 2009). According to the above researchers, a possible explanation may be oxygen deprivation in *Z. mobilis* cells. It has also been accepted that although wild type *Z. mobilis* be strictly fermentative, it possesses a complete redox enzyme system on its cell membrane which rapidly consumes oxygen in an aerobic culture. Consequently, the reduced efficiency of xylose fermentation could be caused by oxygenation competition between *Z. mobilis* and xylose fermenting yeast *P. tannophilus* as presented in current study.

Ethanol production by *Z. mobilis* and *P. tannophilus* using banana peels hydrolysate medium (co-fermentation 2)

In co-fermentation 2, successive inoculation of strains was performed. First *Z. mobilis* was inoculated and *P. tannophilus* was added after 7h fermentation. According to Fu and Peiris (2008), inoculation sequence eliminates the xylose catabolite repression caused by glucose in *P. tannophilus*. Figure 5 reveals that *Z. mobilis* and *P. tannophilus* co-fermentation was more efficient

when strains were inoculated separately on banana peels broth and showed higher ethanol concentration of 11.32 g L^{-1} and productivity (0.60 g L^{-1} h^{-1}) using substrate concentration (15%) and pH 4.5 and 5.0. Moreover it was obtained a higher yield compared with the maximum theoretical yield (77.59%) when compared to co-fermentation 1 (it was obtained 68.81% of the maximum theoretical yields). Further, maximum substrate consumption and biomass in both microorganisms was observed using 15% of banana peels medium at initial pH 5.5 (Table 5). This behavior observed in experiments using pH (5.5) may be due to this pH to be very close to optimal development values for studied microorganisms, what possibly caused modifications in its metabolic route and consequently promoted the use of most of the substrate for growth and synthesis of cellular constituents.

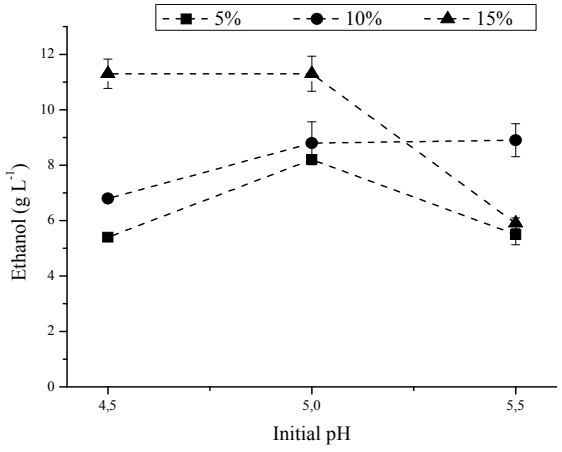

Figure 5. Effect of substrate concentration and initial pH of banana peels hydrolysate medium in ethanol production by co-fermentation of *Z. mobilis* and *P. tannophilus* (co-fermentation 2).

Table 5. Cell biomass, substrate consumed and ethanol productivity observed after fermentation of banana peels medium by co-culture of *Z. mobilis* and *P. tannophilus* (co-fermentation 2).

Substrate concentrations	*Z. mobilis* biomass (g L^{-1})	*P. tannophilus* biomass (g L^{-1})	Sugar consume (g L^{-1})	Productivity (g $L^{-1}h^{-1}$)
pH 4.5				
5%	0.15 ± 0.04	1.28 ± 0.01	20.48 ± 0.15	0.39
10%	0.12 ± 0.02	1.17 ± 0.04	22.67 ± 0.53	0.37
15%	0.28 ± 0.22	1.41 ± 0.07	28.60 ± 1.01	0.60
pH 5.0				
5%	0.02 ± 0.01	1.93 ± 0.02	19.63 ± 0.50	0.43
10%	0.25 ± 0.03	1.58 ± 0.02	22.51 ± 0.53	0.46
15%	0.50 ± 0.03	1.58 ± 0.12	28.61 ± 1.01	0.60
pH 5.5				
5%	0.02 ± 0.01	1.92 ± 0.04	21.32 ± 0.53	0.29
10%	0.22 ± 0.03	1.77 ± 0.03	21.37 ± 2.14	0.47
15%	0.74 ± 0.04	1.92 ± 0.09	30.15 ± 0.94	0.31

The sequential culture of the two microorganisms achieved a high fermentation performance. Co-fermentation (2) was more efficient by ethanol production (11.32 g L^{-1}) when compared to fermentations performed with co-fermentation 1 (4.92 g L^{-1}), *Z. mobilis* (2.90 g L^{-1}) or *P. tannophilus* (4.01 g L^{-1}) separately. This behavior may have occurred due to limitation of xylose consumption by glucose in fermentation medium (Sánchez et al., 2002), (Zhao, Zhang, & Tan, 2008). Since glucose was the preferred substrate, it was consumed before the start of xylose fermentation (Agbogbo et al., 2006). Further, co-culture experiment was started by *Z. mobilis* inoculation on fermentation medium and a part of glucose was metabolized in ethanol and other compounds. When *P. tannophilus* yeast was inoculated (after 7h), the glucose concentration remaining was not sufficient to inhibit xylose metabolism, which was used in ethanol production, increasing its final concentration (Figure 5).

When compared to other studies, the ethanol productivity was also affected by co-culture conditions. The same co-fermentation system was employed by Fu et al. (2009), using a sugar mixture of 30 g L^{-1} glucose and 20 g L^{-1} of xylose, 150 rpm at 30°C , and *Z. mobilis* with *P. stipitis* yeast co-culture. These authors verified that the sequential culture of the two microorganisms increased volumetric ethanol productivity with enhancements from 0.52 to 0.83 g L^{-1} h^{-1}.

Conclusion

Pretreatment followed by enzymatic hydrolysis produced a broth containing 72.80 g L^{-1} (total sugars) and 52.90 g L^{-1} (reducing sugars). Banana peels hydrolysate was efficient to ethanol biosynthesis and the co-culture of *Z. mobilis* and *P. tannophilus* was more effective than individual culture. The best fermentation conditions were initial pH 4.5 and 5.0 and substrate 15%. The highest ethanol production was 11.32 g L^{-1} and productivity reached 0.60 g L^{-1} h^{-1}, with 77.59% of the maximum theoretical yields (co-culture 2) when *P. tannophilus* inoculation occurred after *Z. mobilis*. Results demonstrate that banana peels are an efficient feedstock for ethanol production.

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