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Isolation and Characterization of *Enterobacter sp* Capable towards Tolerating Degradation Products and Fermenting Pentoses

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ABSTRACT

The depletion of fossil fuel resources, in addition to the growing demand for energy, has prompted the development of renewable energies, including bioethanol from lignocellulosic biomass. The acid pretreatment of hemicellulose releases inhibiting compounds in addition to xylose. With the possibility of exploitation of lignocellulose as a fermentation substrate, we isolated an *Enterobacter* characterized by its ability to ferment xylose and tolerate high concentrations of inhibitors. The selection was performed in media containing different carbon and energy sources; glucose, cellobiose, CMC, furfural and 5-HMF. Characterization strategies of the selected strain such as, xylose concentration (from 25 g liter-1 to 100 g liter-1), furfural (0 mM to 25 mM), cell immobilization, were used to quantify the maximum yield of ethanol produced. The results obtained show that our strain can ferment up to 100 g liter-1 of xylose in the presence of 20 mM furfural at 37°C to produce ethanol with a maximum yield of 2.22 g liter- 1 for 24 h under 160 rpm magnetic stirring. The results obtained in this study suggest that the isolated *Enterobacter sp* is a promising strain for the bioconversion of lignocellulosic biomass pretreatment hydrolysate into bioethanol.

Keywords: Enterobacter cloacae, ethanol, furfural, lignocellulose, xylose.

INTRODUCTION

The use of fossil fuels as the main source of energy for most countries has a negative impact on the environment, namely global warming and air pollution (Martins et al., 2019). To address environmental problems and the progressive shortage of combustible feedstocks, great efforts are being made to develop economically viable biotechnology techniques (Modi, Joshi, et Patel, 2018) that would allow the use of fossil fuels to be replaced by the wider use of biomass and renewable sources as a whole, i.e. without limitation of feedstock composition and sources.

From this perspective, it is clear that the biological fermentation of biomass is a great interest as it allows the production of different types of biofuels, such as hydrogen, biomethane, bioethanol, etc. (Mansouri, Rihani, et Bentahar, 2019). There are three types of biofuels. The first type of biofuels is called 1st generation. It is derived from edible biomass, including crops such as maize and sugar cane (Correa et al., 2017). The second one is called 2nd generation. It uses the whole plant and converts renewable compounds from

lignocellulosic biomass into fuel (Didderen, Destain, et Thonart, 2010). The third one is called 3rd generation. It is derived from microalgae biomass (Chowdhury et al., 2019).

The use of sugar or starch feedstocks for bioethanol production is hampered by the availability of resources and agricultural land. Moreover, their use for biofuel production competes with their use in the food sector. The use of lignocellulosic biomass for bioethanol production is an alternative. However, it must be integrated into a concept of total valorization of the plant, of biorefinery by exploiting co-products (e.g. lignin) and by-products (e.g. effluents) (Didderen et al., 2010).

Lignocellulosic biomass is one of the world's most abundant renewable resources (Lorenci Woiciechowski et al., 2020). However, the yield of ethanol production does not yet reach the expected results. On the one hand, the microorganisms used in alcoholic fermentation are unable to produce xylose isomerase (Ogier et al. 1999), namely *Saccharomyces cerevisiae* (Talebnia et Taherzadeh, 2006), which limits xylose fermentation (Camargo Guarnizo et al., 2021). On the other hand, lignocellulose pretreatment releases inhibitory products for fermentation, such as HMF and furfural (Kumari et Singh, 2018; Soares et al., 2020). While improving fermentation efficiency should develop pre-treatment processes (Kumari et Singh, 2018) or look for microorganisms characterized by their tolerance to the high xylose concentrations.

Previous research has identified some xylose-fermenting bacteria and yeasts, the best known of which are: *Spathaspora passalidarum* (Selim et al., 2021), *Pichia stipites* (Lee et al., 1986), *Pachysolen tannophilus* and *Candida shehatae* (Hinman et al., 1989; Kastner, Jones, et Roberts, 1998), *Neurospora crassa* (Dogaris, Mamma, et Kekos, 2013) etc. The major obstacle that limits the use of most of these micro-organisms in industrial processes for the valorization of biomass into bioethanol is their sensitivities to chemical inhibitors (Cadete et Rosa, 2018).

In light of the persistent problems with the fermentation of non-detoxified lignocellulosic biomass, the present study is part of the effort to find new bacteria capable of fermenting xylose, the second most abundant lignocellulose sugar after glucose (Vasylyshyn et al., 2020) and tolerating high furfural concentrations.

METHOD

Bacterial strains, media, and cultivation conditions

Isolated strains from different ecological niches were tested for their ability to utilize glucose, cellobiose, CMC, furfural and 5-HMF as a source of carbon and energy in Wickerham's (1951) medium at concentrations in the range of 0.3-0.5%. A second xylose fermentation test according to the Durham method was applied to those strains that showed the ability to use the different carbon sources mentioned above. Among the strains tested, only one bacterium was selected for the different experiments. This was grown in a Luria-Bertani medium consisting of Peptone 10 g/Liter, yeast extract 5 g/Liter, NaCl 10 g/Liter and xylose 25 g/Liter as carbon source.

Preparation of alginate beads

The immobilized cells were prepared according to the experimental protocol described by Vuillemard et al (1988). The cell pellet obtained after centrifugation was suspended in saline and mixed with a sodium alginate solution to give a final concentration of 2% (w/v). The resulting suspension was added dropwise to a 3% (w/v) $CaCl_2$ solution to create 2-3 min diameter capsules, solidified for 30 min in the same solution. The resulting calcium alginate gel beads were thoroughly washed with sterile distilled water. The initial number of the colony forming units (CFU) in each bead was 2.7 E7.

Conditions of fermentation

The inoculum prepared from isolated agar colonies was incubated overnight at 37°C on a shaker set at 160 rpm. 500 ml shaker flasks were filled with 200 ml of LB (liquid medium), as the description above, containing different concentrations of xylose; 25 g/L, 50 g/L, 75 g/L and 100 g/L. 200 µl of inoculum, with an initial optical density of 1.05 at 620 nm, was added to the shaker flasks to start the fermentation. For immobilized cells, we used a single concentration of xylose (50 g/L). In both cases (free and immobilized cells), the fermentation solutions were sterilized by filtration and incubated in a shaker at 37°C and 160 rpm for free cells and 100 rpm for immobilized cells (Talebnia et Taherzadeh, 2006).

Determination of physiological parameters

During fermentation, exponential growth rates were determined by log-linear regression of optical density 620 versus time with growth rate as the regression coefficient (Sonderegger et Sauer, 2003). The specific biomass yield (Yx/s) was determined from a linear regression coefficient plot of biomass concentration (X) versus substrate concentration (S) during the exponential growth phase (Sonderegger et Sauer, 2003). One unit of absorbance corresponds to 0.7 g litre-1 of dry biomass. During the fermentation, samples were taken regularly throughout the experiments to determine cell growth, the amount of xylose consumed, and ethanol produced.

Analytical methods

The concentration of xylose is determined according to the method of Lorenz Miller (Miller 1959). The determination of ethanol is carried out by GPC using the internal standard method (10 g/L propionic acid prepared in 0.2 N sulfuric acid solution) according to the procedure described by J.R Mouret (2006).

RESULT AND DISCUSSION

In this study, we isolated more than 100 strains (bacteria and yeast) from several ecological niches; pomace, olive pomace, gas station soil, and old sawdust. All these strains were tested for their ability to grow in media containing one of the following carbon sources; glucose, cellobiose, CMC, furfural and 5-HMF and to ferment xylose (data not shown). The bacterial strain isolated from the soil of a petrol station has the ability to grow on the different media mentioned above and produces a considerable volume of gas during xylose fermentation.

Phylogenetic analysis

In a preliminary step, the isolated strain was biochemically characterized before being genetically identified. After extraction of the genomic DNA from cell pellets, the 16S rRNA

gene was amplified by PCR according to the protocol described by (Long et al., 2010). The 16S rRNA gene sequence was aligned using the Basic Local Alignment Search Tool for nucleotides (BLAST) algorithm and compared with existing nucleotide sequences in the GenBank database. Multiple sequence alignment and phylogenetic distance calculation were performed using the CLUSTALW algorithm. Finally, MEGA- 7 software was used to construct the phylogenetic tree while determining the relatedness of the isolated bacteria to other strains in the database.

Selection of efficient strain in bioconversion of xylose to ethanol

Among the microorganisms isolated, several bacteria were selected for their ability to produce ethanol from xylose; among these bacteria studied: *Bacillus endophyticus*, *Klebsiella aerogenes*, and *Enterobacter* sp. with a yield of 1.93 g litre-1, 1.8 g litre-1 and 2 g litre-1 respectively (data not shown). Preliminary ethanol analysis is performed using the corresponding kit.

Xylose metabolism

As we all know, most bacteria do not use xylose as a carbon source (Kawaguchi et al., 2006), whereas some strains such as *Enterobacter* sp. CN1 (Long et al., 2010), *Enterobacter asburiae Strain JDR-1* (Bi, Rice, et Preston, 2009), *Klebsiella pneumoniae* (Nishikawa, Sutcliffe, et Saddler, 1988) etc., are known to ferment xylose into ethanol. This paper describes the effect of xylose concentration (25, 50, 75 and 100 g/L) on ethanol production by *Enterobacter sp*. Table 1 shows the consumption of xylose as a function of time.

In the case of 25 g liter-1, 97.12% of xylose is consumed after 31 h of incubation, whereas the bacteria require 48 h to consume 89.98% and 78.86% for the 50 and 75 g/L initial substrate concentrations respectively. When *Enterobacter sp* is incubated in a medium containing 100 g litre-1 of xylose, degradation becomes slow and more than 48% of the initial substrate remains unconsumed after 72 h of incubation.

Table 1. Consumption of xylose by *Enterobacter sp.*

Xylose	**Time (h)**									
(%)	0	4	6	8	14	24	31	48	56	72
25	25,05	23,34	23,12	20,79	16,21	10,30	0,72	0,64	0,26	0,55
50	48,77	46,91	46,76	46,50	43,19	24,27	19,68	05,01	04,70	04,50
75	73,92	59,62	58,69	53,34	46,33	29,56	25,88	15,85	15,84	14,48
100	99,16	85,45	88,19	88,84	86,28	79,48	75,91	58,87	53,77	48,50

Cell growth

The growth curve of *Enterobacter* sp shows three phases (Fig. 1): The first phase is recorded during 24 hours of fermentation and is characterized by a rapid growth of the microbial biomass, which develops from 0.1 g litre-1 to 1.44, 1.44, 1.39 and 1.27 g litre-1 respectively for the four concentrations studied 25, 50, 75 and 100 g litre-1. The beginning of the second phase (from 24 to 31 h) is characterized by a slight decrease in the biomass "x" for all concentrations used. During the third phase, the biomass produced stabilizes until the end of the experiment, with the exception of the biomass produced in the presence of 25 g

litre-1 where the quantity progressively decreases, reaching 1.21 g litre-1 after 72 h of incubation. From the analysis in Fig. 1 it can be appreciated that the biomass produced in the shake flasks at 100 g litre-1 is always lower than that of the other concentrations and only picks up again after 40 hours of growth. At the end of the fermentation, the biomass in these shake flasks becomes equal to that produced in the 75 g litre-1 case (1.34 g litre-1).

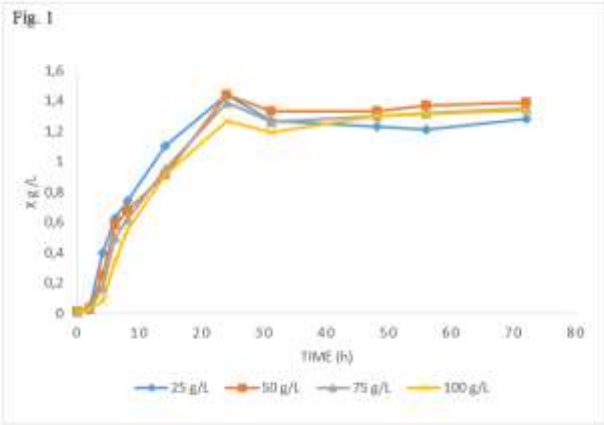

Figure. 1: Growth curves of *Enterobacter* sp.

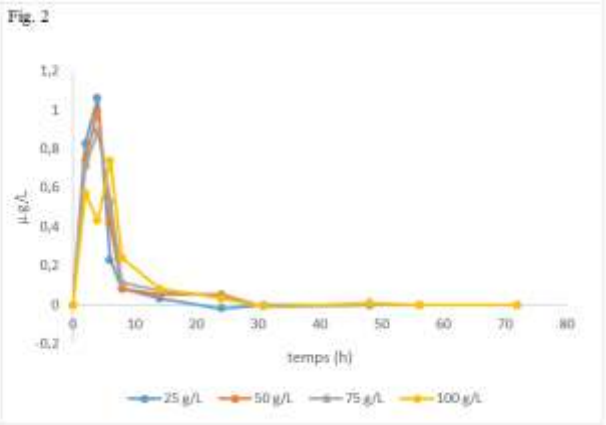

Figure. 2: Effect of xylose concentration on the growth rate of *Enterobacter* sp.

Analysis of this Fig 2. shows that the growth rate "µ" is very high during the first four hours of fermentation and is proportional to the sugar concentration in the culture medium. It can be seen that µmax decreases with increasing xylose concentration. It reaches 1.06 h-1, 1 h-1, 0.89 h-1 and 0.74 h-1 in the 25, 50, 75 and 100 g litre-1 shake flasks respectively (Table 1). After 4 hours of fermentation "µ" decreases rapidly until it becomes zero after 24 hours.

Effect of furfural on bacterial growth

For the inhibitor resistance test, furfural, one of the main inhibitors of several fermenting microorganisms, was chosen (Soares et al., 2020). From Fig. 3 the bacteria grow in the medium as a function of time and inhibitor concentration. It can be seen that there is no difference in growth between the control medium and in the presence of 1 mM furfural, whereas the bacteria require 8 hours, 10 hours and 24 hours of incubation respectively to multiply naturally in culture media containing 5, 10, and 15mM furfural. According to the previous analyses, it has been shown that the bacteria only reach optimum growth after 48 hours of incubation in a medium containing 25 mM furfural. Therefore, the higher the concentration of furfural in the medium, the slower the cell proliferation.

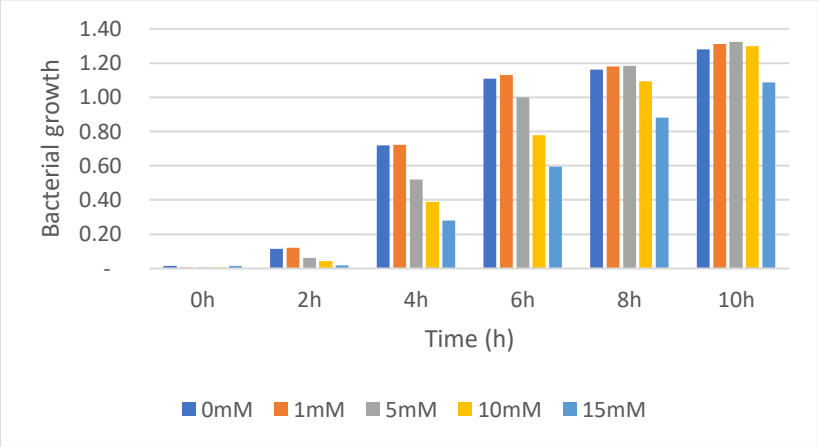

Figure. 3: Effect of furfural on the growth of *Enterobacter* sp

Ethanol production

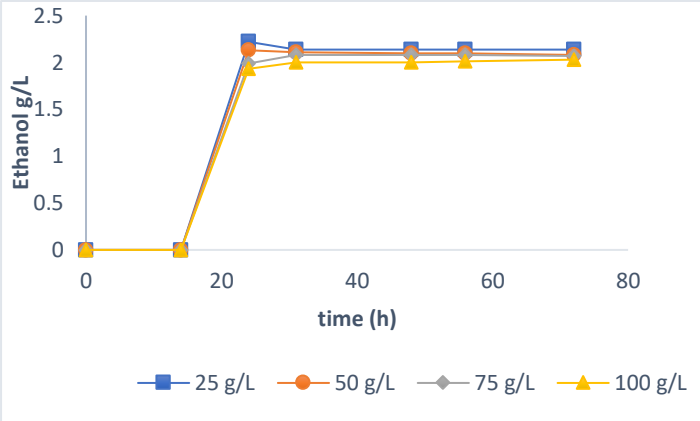

Figure. 4 shows that ethanol only appears in the ethanol production

Culture medium after 24 hours of fermentation in the four shake flasks. After a rapid production in the order of 2.22, 2.13, 1.99 and 1.94 g litre-1, respectively for 25, 50, 75 and 100 g litre-1 of xylose as the only carbon source in the culture medium, the ethanol level in the media stabilizes at 2.14 and 2.1 g litre-1 for 25 and 50 g litre-1 xylose with a slight increase in the other two shake flasks at 75 and 100 g litre-1 in which the yield reaches 2.08 and 2.03 g litre-1 respectively at the end of fermentation. The bacteria ferment xylose (2.22 g litre-1) with a maximum yield of 0.15 g g-1 (24.59% of the theoretical yield).

Effect of furfural on yield

The previous result led us to test the capacity of xylose assimilation and fermentation by the bacteria in the presence of high furfural concentrations. Table 2 shows the percentage of xylose consumed as a function of the inhibitor concentration.

Thus, the bacterium was able to consume 9.36 % and 6.7 % xylose in 24 hours at 37 °C in the presence of 15 and 25mM furfural respectively, whereas after 48 hours it was able to assimilate more than half of the xylose in the presence of 15 mM furfural and reached 70.4 % of its reproductive capacity in ethanol, while its capacity to degrade xylose decreased to 34 % in culture medium containing 25 mM furfural with a yield of 57.34 % ethanol compared to the control, Table 2.

Table 2. Effect of furfural on xylose consumption and ethanol yield

Concentration of furfural in mM	Time of incubation (h)	Initial xylose (g.litre-1)	% of xylose consumed	% of ethanol produced
15	24	50	09,36	-
	48	50	50,41	70.40
25	24	50	06,70	-
	48	50	34,42	57.34

Our strain belongs to the Enterobacteriaceae family, known for their ability to produce several valuable molecules from the degradation of pentose and hexose sugars from lignocellulosic biomass. They are often used to produce 2.3-BD (Li et al., 2015; Wang et al., 2012; Zhang et al., 2016), biohydrogen (Choonut, Saejong, et Sangkharak, 2014; Pachapur et al., 2017), while studies on bioethanol production by this type of bacteria are still scarce (Sarkar et al. 2019), despite the distinguished ability of *Enterobacter* sp. to assimilate a wide range of carbon sources (Wang et al., 2012; Xu et al., 2012). The isolated *Enterobacter* sp. was able to grow on several carbon sources (glucose, cellobiose, CMC, furfural and 5-HMF), thus it is not very demanding with respect to environmental conditions. *Enterobacter* sp showed a high efficiency of xylose assimilation with a consumption rate of more than 50% after 24 hours of incubation, for substrate concentrations up to 75 g liter-1. After 72 hours of incubation almost all xylose is consumed.

The growth curve of the bacterial strain shows that the biomass development has three main phases:

a) The first is a growth phase.

b) After 24 hours of incubation, the second phase of mortality is identified; due to the sudden change in the physic-chemical parameters of growth. This stage is characterized by a decrease in biomass following the accumulation of ethanol, causing an increase in ATP requirements, without necessarily being associated with a decrease in the rate of xylose consumption (Garrigues et al., 1998).

c) The third phase, the so-called maximal stationary phase, is ensured by a metabolic adaptation that arises to protect bacterial cells from total degradation. The cells remodel their catabolism and anabolism to produce intermediate metabolites, reducing power and energy. On the other hand the strain showed maximum growth at a temperature of 37°C, this seems to be favorable in reducing the possibility of contamination compared to fermentation at low temperatures (Wang et al., 2012).

In addition, the growth and productivity of micro-organisms is influenced by the unavoidable presence of furfural in lignocellulose hydrolysates after acid pre-treatment (Gutiérrez, Ingram, et Preston, 2006). The study of the effect of this inhibitor showed that it inhibits all cell growth at low concentrations ranging from 7 to 25 mM (Allen et al., 2010), further work by Zaldivar et al in 1999 reported that a concentration of furfural between 5 and 20 mM can alter the growth and metabolic rates of E. coli LY01 (Zaldivar, Martinez, et Ingram, 1999). G. Gong et al., (2015) reported that a strain of Enterobacter cloacae GGT036 was able to overcome up to 35 mM furfural (but the study does not give further details) (Gong et al., 2015). In this research, the effect of several concentrations of 1-25 mM furfural was

studied, and it was found that the strain is able to multiply naturally in concentrations up to 15mM. In the range of 1-10 mM furfural biotransformation is simultaneous with cell growth, whereas it shows a delay in growth in the higher concentrations. Similar results were found by T. Gutierrez et al., (2002) working with an *E. coli* strain on LB medium. It can be said that the strain can reduce furfural in a short time and thus resume its natural growth if the concentration of furfural is low in the medium. However, the delayed cell growth in concentrations above 25mM can be explained by the low production of the catalyzing enzymes this biotransformation, which is a direct result of the low amount of the initial inoculum (Gutierrez, Ingram, et Preston, 2002).

The strain in the experiment was examined for its ability to ferment xylose. The results obtained indicate that the rate of ethanol produced varies slightly with the concentration of xylose, thus the strain produced ethanol at a maximum yield of 2.22 g litre-1 for 25 g litre-1 of xylose. Similar results were reported by Lixiang li et al in 2014 by a wild type strain of Enterobacter cloacae with a yield of 2.8 g liter-1 from 20 g litre-1 in 14 hours where the bacteria consumed 15 g litre-1 or 73% of the initial substrate (Li et al., 2015). With the same strain of Enterobacter cloacae, D. Sarkar et al. obtained 3.07 g litre-1 of ethanol after 18 hours of fermentation of dried banana peels (Sarkar et al., 2019). While the maximum yield achieved by the strain in the present study was in 24 hours after consumption of 58.88% xylose. The aforementioned results of Sarkar et al showed a decrease in the amount of ethanol in the culture medium after 18 hours of incubation, which we did not notice in our work, as the alcohol level remained almost constant in the culture media over time. The amount of substrate not fermented in the case of high xylose concentrations is either due to toxicity of the medium, or to inhibition of the product in collaboration with the high sugar concentration or by excess substrate.

Table 3. Growth and production kinetics

Parameters studied	**Concentration of xylose (g/L)**			
	25	50	75	100
P_{max} $g_p.L^{-1}$	2.22	2.13	2.08	2.03
% sugar fermented	58.88	50.24	60.01	19.84
rs (g. $L^{-1}.h^{-1}$)				
qp ($g.g^{-1}.h^{-1}$)	0.064	0.061	0.059	0.063
Qp (g. $L^{-1}.h^{-1}$)	0.093	0.089	0.083	0.08
Yp/s ($g_p.g_s^{-1}$)	0.15	0.09	0.04	0.1
Yx/s ($g_x.g_s^{-1}$)	1.01	2.2	0.35	0.14
Yp/x ($g_p.g_x^{-1}$)	2.68	1.69	1.88	2.23
theoretical yield	0.61			
% of theoretical yield	24.59	14.75	6.56	16.39
m	1.43	1	20.01	37.1
Yield = Yp/s /0.511 x100	29.5	17.01	8.78	19.21

The ethanol yield does not increase with increasing xylose concentration, on the contrary, it decreases by 29.5, 17.07 and 8.78 % respectively for the three concentrations used 25, 50 and 75 g. litre-1, this result is explained by the decrease of Pmax from 2.22 to 2.08 g litre-1 respectively for 25 to 75 g litre- 1 with a maximum substrate-product conversion rate Yp/s of 0.15 g. g-1 in a medium containing 25 g litre- 1 of xylose. The specific rate of ethanol production qp decreases with increasing substrate, ranging from 0.064 to 0.01 g. g. h-1 for 25 and 75 g. litre-1. Furthermore, *Enterobacter* sp. shows acceptable productivity rates for xylose with a productivity of up to 0.093 g. L-1 h-1 (Table 3). These results are due either to the inhibitory effect of the substrate or to the existence of a redox threshold that limits the bioconversion of the substrate into ethanol. These two hypotheses are elucidated by the increase in energy requirements reflected by the increase in maintenance energy, which increases as a function of the xylose concentration, from 1.43 for 25 g litre-1 to 20.01 in the case of 75 g litre-1.

Previous work has shown that the presence of furfural causes a delay in production (Gutierrez et al. 2002) and a significant decrease in yield (Soares et al., 2020). For the same inhibitor, almost similar effects were found for *Enterobacter* sp. where ethanol appeared only in trace amounts within the first 24 hours and reached 73.4% 57.34% of control (no furfural) after 48 hours incubation in media containing 15 and 25mM furfural respectively (Table 2).

We note that in the immobilized cells, no improvement in ethanol production was noted compared to the free cells. The values obtained by the immobilized cells allowed us, although lower than those obtained by the free cells, to use them in the fermentation of lignocellulosic biomass, which releases compounds that inhibit the growth of the free microorganisms used for alcoholic fermentation (Lopez et al., 2004).

CONCLUSION

Microorganisms capable of simultaneously fermenting all the sugars in the lignocellulosic hydrolysate and enduring high concentrations of inhibitors are highly sought after by biorefineries because of their properties, which surpass those of the yeasts used in second-generation bioethanol production. The results obtained show that the strain in the present study ferments xylose with an ethanol yield of 2.22 g litre-1 and tolerates 25 mM furfural. Thus, it is an encouraging strain for the fermentation of lignocellulose hydrolysate. In addition to the production of ethanol, the engineering strategy using *Enterobacter* could also be used in the production of other valuable chemical products using sugars derived from lignocellulosic biomass. Efforts should be directed towards identifying stress and ethanol enduring microorganisms using a wide range of carbon sources to produce appreciable (considerable) amounts of the product.

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