



Biocatalytic transformation of furfural into furfuryl alcohol using resting cells of *Bacillus cereus*

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ARTICLE INFO

Keywords:

Furfural
Furfuryl alcohol
Dehydrogenase
Biocatalysis
Bacterium
Resting cells

ABSTRACT

The bioconversion of furfural to furfuryl alcohol is an attractive route in biomass valorization that could replace traditional contaminant methods. The use of whole cells has been explored for this purpose. *Bacillus cereus* without previous treatment with furanic compounds was used to selectively obtain furfuryl alcohol. Growing and resting cells were employed. Using growing cells of *B. cereus*, lower yields to alcohol were obtained because of furfural toxicity. However, employing resting cells it was possible to reach higher yields to furfuryl alcohol. Optimal operative conditions were studied: different concentrations of furfural, glucose and molybdenum, pH, and temperature. Thus, glucose (100 mM) and molybdenum (0.1 mM) were added to maintain cell biomass obtaining a yield to furfuryl alcohol close to 80% at 30 °C, pH 7.2 from 30 mM of furfural.

1. Introduction

Furfural (FAL) is recognized as a platform molecule derived from lignocellulosic biomass, and it is a compound commercially important for the production of chemical products such as 2-furoic acid (FA), furfuryl alcohol (FOL), levulinic acid, furan, and 2-methylfuran [1]. The main transformation involves the reduction or oxidation of the carbonyl group to obtain FOL and FA, respectively. FOL is the most important derivative of FAL [2] and is obtained by FAL reduction using Cu-Cr catalysts [3,4]. However, the use of chromium and the formation of 2-methylfuran and furan as by-products have led to the search for new methodologies for its synthesis [5]. In this sense, the production of FOL via biocatalysis has been recognized as a promising alternative due to the elimination of contaminating reagents while retaining the selectivity of the reaction [6].

The oxidation/reduction reactions constitute the initial step of the biological pathways for the degradation of FAL. Although furanic aldehydes are highly toxic to microorganisms [7], some microorganisms have clusters of genes or unique genes whose expression is activated in the presence of these compounds [8,9]. In both aerobic and anaerobic microbes, the most common native mechanism observed for detoxifying FAL employs cofactors (e.g., NADH or NAD⁺) to convert the carbonyl group into the corresponding alcohol or acid [10]. Thus aldehyde reductases, aldehyde dehydrogenases, and alcohol dehydrogenases are involved in the biotransformation of this type of furan aldehyde (Fig. 1). Whole cells are preferred for transformation processes over isolated enzymes because cells are economical, more stable, and do not require complex regeneration systems [11].

The tolerance to FAL seems to be an essential factor in the conversion of higher FAL concentrations. Various studies have reported the

Abbreviations: FAL, Furfural; FOL, Furfuryl alcohol; FA, Furoic acid; ALENB, Nutrient broth; NA, Nutrient agar.

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<https://doi.org/10.1016/j.cattod.2021.01.011>

Received 20 May 2020; Received in revised form 1 December 2020; Accepted 22 January 2021

Available online 3 February 2021

0920-5861/© 2021 Published by Elsevier B.V.

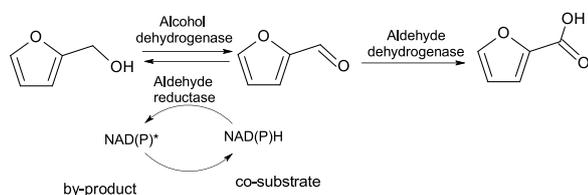


Fig. 1. Scheme of the biotransformation of furfural using whole cells.

biotransformation and fermentation of FAL into FOL using microorganisms [2,12–15]. Furan toxicity can be overcome by increasing the biomass density. Thus, higher initial biomass concentration implies that more enzymes are present initially converting into the less toxic compound, resulting in apparently increased furanic aldehyde tolerance [16]. Other strategies such as the regeneration of cofactors have allowed the increase of FAL tolerance in microorganisms. Thus whole cells of *B. coagulans* NLO1 present a 96% yield from 42 mM of FAL using glucose as co-substrate [12]. Glucose serves to regenerate the NADH cofactor, and at the same time is converted to lactic acid using a biphasic system leading to 86% FOL yield from 208 mM of FAL [17]. In fact, glucose has also been used as co-substrate to improve the bioconversion of FAL and hydroxymethylfurfural in yeasts [2,18]. *Meyerozyma guilliermondii* SC1103 cells tolerate up to 200 mM of FAL with a yield to FOL of 96% [2], but with immobilized cells the yield to FOL decreases slightly to 81%. Therefore NAD(P)H dependent alcohol dehydrogenases catalyze the reduction of FAL, while glucose as a co-substrate is enzymatically oxidized for generating NAD(P)H catalysts [2].

On the other hand, *B. cereus* has been reported previously as a strain that degrades FAL up to a concentration of 40 mmol/L; the best FAL degradation ability (35%) occurred in long periods (7 days). However, the authors did not establish the products formed or the possible FAL adsorption in this sporulating microorganism [19]. *B. cereus* is a gram-positive, facultative anaerobic rod-shaped endospore-forming bacterium [20–22]. The mechanism of sporulation of *B. cereus* has been described by various authors [20,21]. In all cases, it has been observed that its sporulation begins at the end of the late stationary phase [21]. Although *B. cereus* has been tested in distinct organic transformations [23–25], none of these studies correlate spore formation with the yields obtained. Besides, *B. cereus* is a typical bacterium that expresses glucose dehydrogenase even after exponential growth is completed and sporulation has started. [26] Glucose dehydrogenase from *B. cereus* has been used to exploit its ability to reduce prochiral ketones stereoselectively to chiral alcohols [27,28].

In this work, a strain of *B. cereus*, isolated from soils without previous treatment with furanic aldehydes, was grown in the presence of FAL. The ability of the bacterium to convert this compound was evaluated. Later, resting cells of *B. cereus* in different conditions were assayed until optimizing FOL production. The effect of glucose to regenerate NAD(P)H in this bacterium was studied, thus as the addition of Mo⁶⁺ as a cofactor of oxidoreductases involved in the transformation of furfural.

2. Materials and methods

2.1. Materials

The following reagents and culture media were purchased from commercial sources and used without further purification: Nutrient agar (NA, HiMedia ref. M561), nutrient broth (NB, Scharlau ref. 02,140,500), furfural (Aldrich, 99%), furfuryl alcohol (Aldrich, 99%), furoic acid (Aldrich, 99%), malachite green oxalate (Merck, 98%), sodium monohydrogen phosphate (Merck, 99%), sodium dihydrogen phosphate (J.T. Baker, 99%).

2.2. Microorganism

The Laboratory of Vegetal Physiology at Universidad Pedagógica y Tecnológica de Colombia provided a *B. cereus* strain isolated from soils not treated with furanic aldehydes. This strain was cultured in NA solid medium containing (g L⁻¹): peptone (5.0), beef extract (3.0), agar (15), and 30 mM of FAL as a carbon source, and incubated at 30 °C [29].

Single colonies were cultured in modified NB liquid medium containing (g L⁻¹): yeast extract (2.0), meat extract (1.0), and sodium chloride (5.0), and incubated at 30 °C overnight with agitation. The purity was verified by BBL Crystal GP identification system and microscopy observation previous to Gram staining. The strain was conserved in 30% glycerol at -80°. To verify the identity, single colonies were inoculated in NB and grown overnight; 1 mL of bacterial culture was used to extract genomic DNA and to amplify the partial sequence of the 16S rDNA gene. The similarity tree was generated using the Neighbor-Joining method (Fig. S1) [30].

Samples of the NB were taken every 2 h for 24 h to quantify the total number of viable cells and construct the growth curve (Fig S2). Serial dilutions in sterile water of each sample and dilutions of 10⁻⁸, 10⁻⁹, and 10⁻¹⁰ were plated on NA, and the plates were incubated at 30 °C. The results were expressed in log of colony forming units (CFU log) per mL.

2.3. FAL bioconversion using growing cells

FAL concentration effect (0–75 mM) on growth rate (μ) of *B. cereus* was studied on cultures growing in modified NB medium, pH 7.2, 30 °C, and 250 rpm, using an initial optical density at 600 nm (OD₆₀₀) of 0.05 or 1.9 × 10⁹ CFU/mL. A Whittaker ELX808 spectrometer with flat-bottom 96-well cell culture plates (Nest) was used to measure OD₆₀₀. In each well, 200 μ L of growing bacteria was added. The specific growth rate (μ_{max}) was calculated from the steepest part of the ln(OD₆₀₀) curve. The concentration of 30 mM was chosen to analyze the conversion and yield to FOL and FA until 24 h. The viable cells were determined by plating in medium containing 30 mM of FAL. The presence of spores was determined by staining with 5% malachite green aqueous solution.

2.4. Biocatalysis using resting cells

The assays using resting cells involved a culture of this strain in NA overnight. The growing cells were resuspended in 5 mL of buffer phosphate at pH 7.2 until obtaining an OD₆₀₀ of 1.0 or 200 ± 10 × 10¹⁰ CFU/mL. The cells in this OD₆₀₀ remained in stationary phase (Fig. S3).

Four treatments were assayed to analyze the bioconversion of FAL. First, concentrations from 30 to 200 mM of FAL were assayed at pH 7.2, 30 °C, and 250 rpm. The FAL concentration with the highest conversion and yields was used in the next experiments. Second, different concentrations of glucose (0–150 mM) as co-substrate were assayed, using 30 mM of FAL and keeping the pH, temperature, and rpm constant. Third, an assay involving the effect of Mo⁶⁺ using Mo(SO₄)₃ on the conversion of FAL was performed. In the experiment the concentration of Mo⁶⁺ was varied from 0.1 to 0.8 mM using 30 mM of FAL, pH 7.2, and 30 °C. The last treatment evaluated was the effect of glucose (100 mM) with Mo⁶⁺ (0.1 mM). In this treatment temperatures ranging from 25 to 35 °C, and pH ranging from 5.2–9.2 were studied using phosphate buffer. Each treatment was performed in triplicate. The percentage of viable cells for each treatment was expressed as the ratio between CFU/mL after 12 or 24 h over the initial CFU/mL.

2.5. Analytical methods

FAL, FOL, and FA were quantitatively determined by high-performance liquid chromatography using an apparatus Knauer Azura equipped with a Waters C-18 column. The column temperature was kept constant at 35 °C, and as mobile phase water/acetonitrile (80:20) with a flow rate of 0.4 mL/min was used. FAL, FOL, and FA concentrations

were determined using the response factors obtained by the UV detector at 230 nm [31]. Product yields (Y) were calculated as the ratio of the product concentration to the initial FAL concentration.

3. Results and discussion

3.1. Bioconversion of FAL on growing cells

The microbial conversion of furanic aldehydes is strongly related to their concentration due to the inhibition effect on cell growth. The effect of different concentrations of FAL on *B. cereus* cells was studied using the specific growth rate (μ_{\max}) calculated from the $\ln(\text{OD}_{600})$ curve at each concentration. This approximation using OD_{600} values could lead to mistakes. However, as the linearity between OD_{600} and CFU/mL is maintained in the logarithmic phase, OD_{600} values were used (Fig. S2). There is an exponential decay of the specific growth rate of bacteria consistent with $\mu = \mu_0 \exp(-\lambda [\text{FAL}])$, where λ is the substrate inhibition constant (Fig. 2a). The growth rate drops drastically in *B. cereus*, indicating a strong inhibition effect of FAL.

Fig. 2.b displays the same conversion of 30 mM of FAL and yield to FA and FOL as the viable cells (CFU/mL). There is a good correlation with the OD_{600} obtained at 14 h. The cells begin to convert FAL in the exponential phase after a prolonged lag phase, as has been described by other authors using other bacteria [32]. However, after 16 h cell death begins, and the data observed by optical density are erroneous. Using malachite green stain, spores at 24 h were evidenced, thus FAL allowed sporulation after its conversion. After cellular death, the yields to FA and FOL do not increase, but the conversion of FAL increases. This result is probably a consequence of the transformation of FAL by other substances present in the culture medium acting as biocatalyst.

When the effect of each component of the culture medium on FAL conversion was analyzed, it was found that peptone was responsible for almost 20% of the conversion obtained in 24 h (data not shown). The effect of peptone on the conversion of organic compounds was also reported by Hellauer et al. [33]. They argued that the transformation of organic chemicals was favored under redox conditions. However, in this assay the presence of peptone did not favor the selectivity to FA or FOL.

Peptone in the medium affects FAL conversion, and FAL inhibition of the growth rate of *B. cereus* limits the practical applications via fermentation. So, we decided to continue the experiments using resting cells because higher initial biomass could increase the conversion of FAL since they are not used for biomass production.

3.2. Bioconversion of FAL on growing cells

At this point, *B. cereus* was grown in AN medium until obtaining a

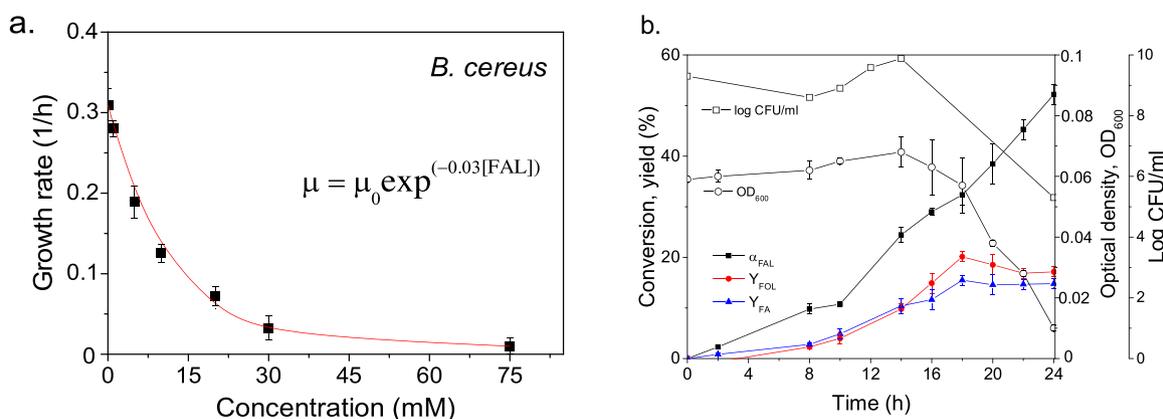


Fig. 2. (a) Effect of concentration of furfural on *B. cereus* indicated as the growth rate of the bacterium in modified nutrient broth and different concentrations of FAL (mM), initial OD_{600} 0.05, 30 °C, pH 7.2, and 250 rpm. (b) OD_{600} , Log CFU/mL and Conversion/Yield versus time with 30 mM of furfural to FOL and FA using growing cells in modified nutrient broth.

sufficient amount of bacterial cells in stationary phase. The results in Fig. 3 show that resting cells of *B. cereus* are not capable of converting FAL in concentrations higher than 100 mM. Besides, significant differences in conversion and yields are not observed between growing and resting cells when a concentration of 30 mM is used (compare Figs. 2b and 3 b). FAL caused an earlier cellular death, and as the reaction progressed, sporulation took place as a defense mechanism, and the percentage of the viable cells decreased to 12 h. The conversion before 12 h occurred in the absence of the lag phase. However, when the microorganism begins to use FAL as source carbon, the viable cells increase at 24 h, being not observed a change in yield to FA or FOL.

It is well recognized that glucose used as co-substrate can regenerate NADH [34]. Fig. 4a shows that 100 mM of glucose is necessary to favor the maximum preferential conversion of FAL. An excess of glucose causes a decrease in FAL conversion and the production of FOL. This can be explained considering that glucose is the most common carbon source used in sporulation media, but also the addition of a low concentration of glucose favors biofilm formation, which is associated with a decrease of the pH [22]. The biofilm-forming ability coupled with spore development in *B. cereus* is commonly associated with the mechanism of resistance to toxic compounds [35]. The formation of biofilm observed at high glucose concentrations was evidenced by the increase in the viscosity of the reaction medium and the decrease of pH at 6.0. The values of OD_{600} remained constant, but the number of CFU/mL decreased substantially. This difference could be associated with a higher spore formation. It has been reported that the absorbance increase as a consequence of the phase-bright spore. Furthermore, alanine and inosine are necessary to spore germination [21], it was clearly not found in AN media. In all cases, the yield to FOL did not increase as a consequence of the possible sporulation of *B. cereus* after this time.

Mo^{6+} was studied having an account that the presence of metal cofactors should activate the oxidoreductases and consequently improve the bioconversion of FAL [36,37]. The results of the effect of distinct concentrations of Mo^{6+} on the conversion of FAL without the presence of glucose are shown in Fig. 5. It is interesting to see how the presence of Mo^{6+} salt increases both the conversion and yield to FOL, while the formation of FA is inhibited. Yield to FOL close to 90% was obtained using 0.8 mM of Mo^{6+} at 24 h (Fig. 5b). Besides, in every concentration of Mo^{6+} studied, viable cells decreased drastically at 24 h. OD_{600} values decrease in the same trend. Despite death cellular observed, it seems that the presence of Mo^{6+} in resting cells of *B. cereus* uses a molybdenum-dependent dehydrogenase which catalyzes the reduction of different substrates [38–40].

The effect of FAL concentration was studied using 0.1 mM of Mo^{6+} and 100 mM of glucose. These conditions were chosen with the aim of decreasing the amount of Mo^{6+} used and favor the regeneration of

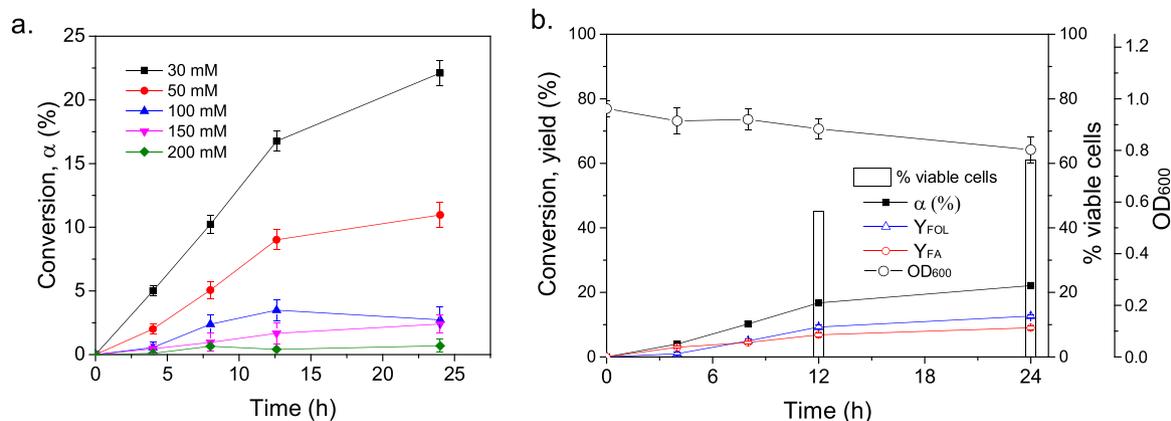


Fig. 3. (a) Conversion at distinct concentrations of furfural using resting cells of *B. cereus*. (b) Viable cells (%), OD and Conversion/Yield versus time using a concentration of 30 mM of furfural. General conditions: OD_{600} 0.96, 5 mL of phosphate buffer solution, pH 7.2, 30 °C, and 250 rpm.

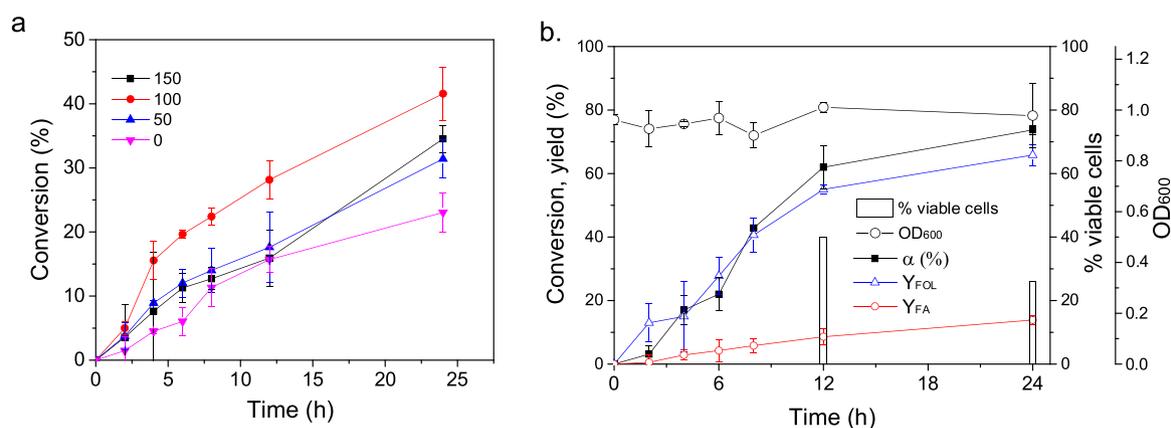


Fig. 4. (a) Effect of glucose concentration (mM) on the conversion of furfural using resting cells of *B. cereus*, (b) Viable cells (%), OD and Conversion/Yield versus time with 100 mM of glucose. General conditions: 30 mM of FAL, OD_{600} 1.0, 5 mL of phosphate buffer solution, pH 7.2, 30 °C, and 250 rpm.

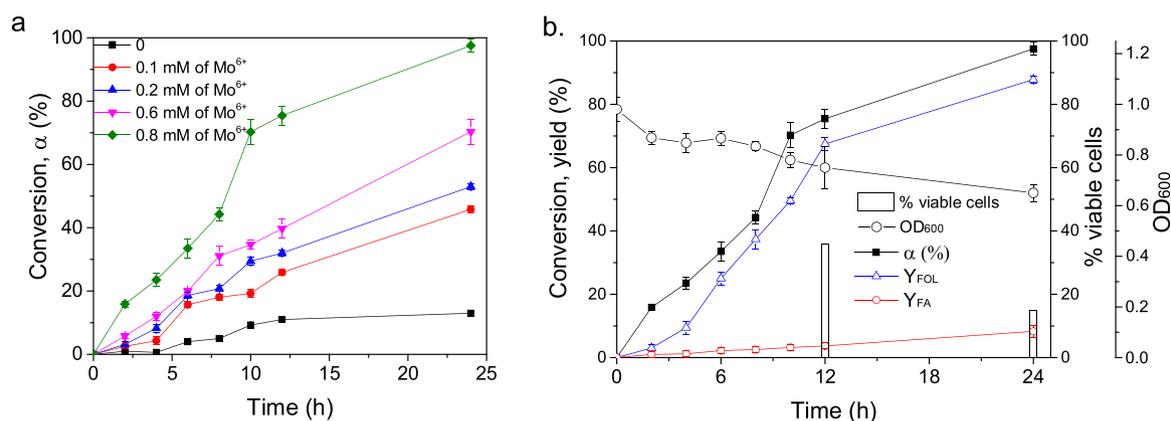


Fig. 5. (a) Effect of Mo^{6+} on the conversion of furfural using resting cells of *B. cereus*, (b) Viable cells (%), OD_{600} and Conversion/Yield versus time with 0.8 mM of Mo^{6+} . General conditions: 30 mM of FAL, OD_{600} 1.0, 5 mL of phosphate buffer solution, pH 7.2, 30 °C, and 250 rpm.

NADH via glucose dehydrogenase, assuming that this enzyme is expressed in exponential growth and sporulation [26]. The results are shown in Fig. 6a. With an initial concentration of 30 mM of FAL, the conversion was 80%, but with 50 mM only it was close to 40%. This indicates that the bacterium is only capable of metabolizing low concentrations of FAL due to higher requirements for NADH regeneration. Under this treatment at 24 h the viable cells increased with respect to previous treatments with only glucose or Mo^{6+} . Unfortunately, the yield

to FOL is minor compared with only Mo^{6+} (0.8 mM).

Fig. 7a displays the temperature effect on the conversion and yield to FA and FOL. The optimum temperature was 30 °C. At the lowest and highest temperatures, the yield to FOL decreased slightly. This loss of activity can be explained considering the alcohol dehydrogenase denaturation. Although it has been reported that alcohol dehydrogenases act in the 25–45 °C range of temperature [41], in this work, the enzymes of *B. cereus* involved in FAL biotransformation worked at an optimum

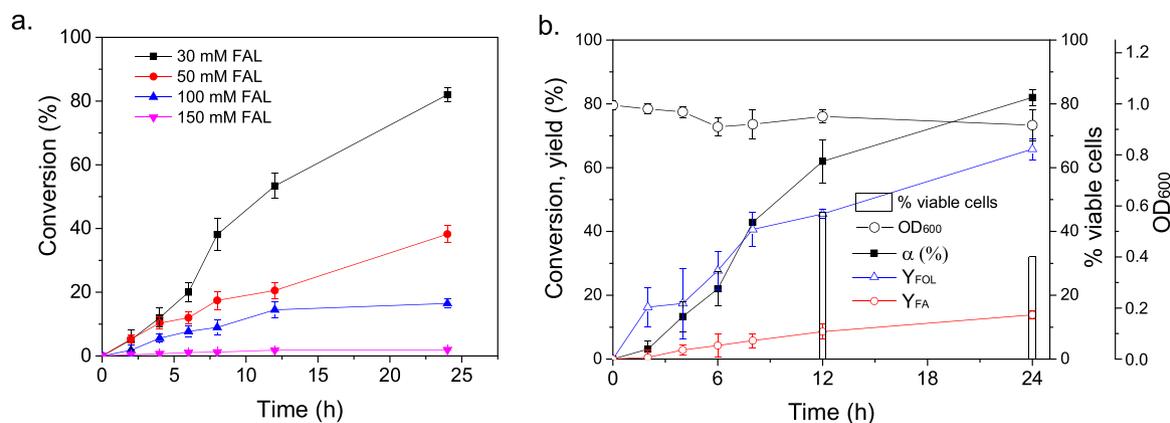


Fig. 6. (a) Effect of furfural concentration on the conversion of furfural, and (b) Viable cells (%), OD and Conversion/Yield versus time with 100 mM of glucose, 0.1 mM of Mo⁶⁺, OD₆₀₀ 1.0, 5 mL of phosphate buffer solution, pH 7.2, 30 °C, and 250 rpm.

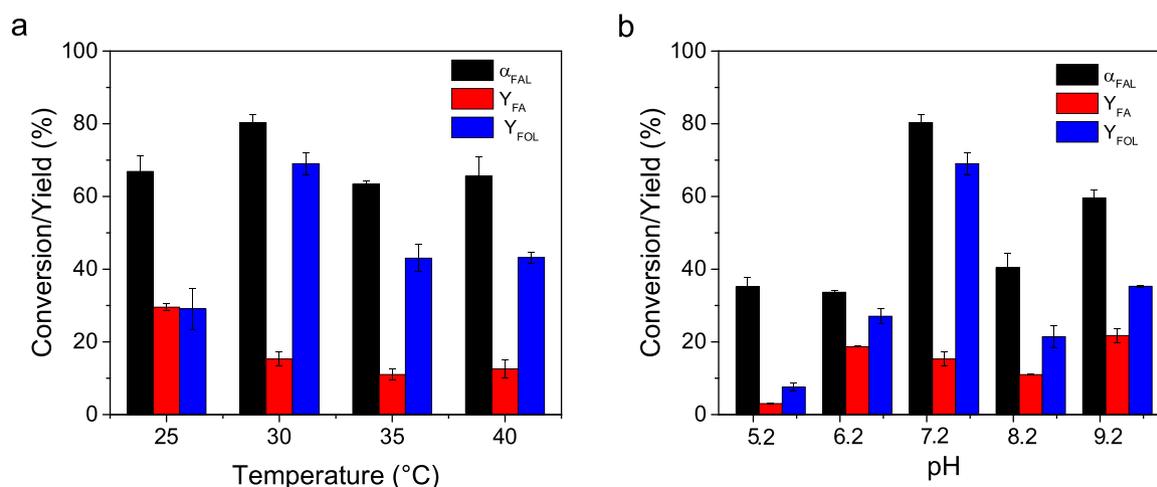


Fig. 7. (a) Effect of temperature, and (b) pH on furfural biotransformation using *B. cereus*. Reaction conditions: 30 mM FAL, OD₆₀₀ 0.9, 5 mL of phosphate buffer solution, 250 rpm, 100 mM of glucose and 0.1 mM Mo⁶⁺, 24 h.

temperature of 30 °C. Phosphate buffer at pH 7.2 gave the best conversion of FAL favoring glucose dehydrogenase activity, increasing the metabolism and ATP production to get a higher conversion [42] (Fig. 6b). However, a better yield to FOL was observed in a narrow pH range. Similarly, this effect of pH on the selectivity was observed with wild-type *C. testosteroni SC1588* that contains 3-succinoylsemialdehyde-pyridine dehydrogenase (SAPDH) [43]. On the other hand, the highest ratio of FA/FOL occurred at high pH values, at which it is commonly observed an optimal reduction of NAD⁺ [42].

Enzymes such as aldehyde oxidases, dehydrogenases, and aldehyde reductases have been reported to be involved in the detoxification of aldehydes in alcohols [2]. None of the cluster genes [44] or individual genes [45–47] reported to be involved in oxidation and reduction reactions of FAL were found in *B. cereus* genome. Therefore the plasticity of the bacterium can be responsible for the phenotypic response due to changes in source carbon that affect their metabolism. Moreover, in *B. cereus* the presence of carbonyl reductase and aldehyde dehydrogenase presenting bidirectional oxidizing and reducing activities has been reported [24,48]. The primary results in this work indicate that Mo⁶⁺ ion-dependent enzyme in *B. cereus* could be involved in the selective reduction of FAL to FOL. However, more experimental evidence is still needed to identify the enzyme responsible for this selective reduction.

With regards to FAL tolerance some authors recently reported the conversion of 200 mM of FAL with wild fungi [2,18], with respect to bacteria, *B. coagulans* NLO1 tolerates 208 mM of FAL [17]. *P. putida*

KT2440 tolerated a concentration of up to 20 mM of FAL [10], while *C. thermocellum* 1313 tolerated a maximum 10 mM of FAL [49]. In this work, *B. cereus* produced FOL selectively from 30 mM of FAL using resting cells despite a higher inhibition by the substrate. Besides, it is highlighted that using our strain, which was not genetically modified, similar results to previous reports were obtained.

4. Conclusion

A wild strain of *B. cereus* never grown before in the presence of furanic aldehydes was used to biotransform furfural to furfuryl alcohol. Using growing cells, 40% of furfural conversion was quantified without selectivity toward furfuryl alcohol or furoic acid. While using resting cells, a yield to furfuryl alcohol, highly dependent on the concentration of Mo⁶⁺ salt, was observed. Using 100 mM of glucose and a concentration of 0.1 mM of Mo⁶⁺, high conversion with a yield of 80% can be obtained, maintaining a higher proportion of viable cells.

Funding

This work was financial supported by Vicerrectoría de Investigaciones y Extensión-Universidad Pedagógica y Tecnológica de Colombia. Award 2851

Authors' contributions

JJM and LR conceived and designed the research. AR conducted the experiments. AR, JJM, GPR, MHB, LR, and RL analyzed data and wrote the manuscript. The authors read and approved the final manuscript.

Declaration of competing interest

The authors report no declarations of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.cattod.2021.01.011>.

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