Production of raw starch degrading amylase by B*acillus subtilis* TLO3 and its application in bioethanol production using starch-rich flours

Abstract

Since the 20th century, oil became indispensable in fields of energy and chemical industry, leading to a global dependence and causing great damages to environment. Bioethanol is currently the most widely used liquid biofuel in the world. The starch pre-treatment for ethanol production requires the use of amylolytic microorganisms, or starch degrading enzymes, such as α-amylases and glucoamylases, to convert it into fermentable sugars. In this study, an amylase hyperproducer strain *Bacillus subtilis* TLO3 newly isolated from natural soil, was used for amylase production. The crude enzyme was used thereafter for raw corn and wheat starches pre-treatment. After that, the yeast *Saccharomyces cerevisiae* was inoculated into the saccharified starch solutions for fermentation. The total reducing sugars released during saccharification were measured, and the amount of ethanol produced, as well as, the reducing sugars were monitored all along the fermentation process. Thus, 70% and 91% reducing sugars were obtained after saccharification of wheat and corn starch, respectively, by *B. subtilis* TLO3 amylase. The fermentation process monitoring showed a continuous decrease in the total sugars, concurrently with an increase in ethanol production that reached 0.92 g/l (2%) for wheat flour and 1.1 g/l (2.4%) for corn flour after 24 h.

Keywords: amylase; *Bacillus subtilis* TLO3; bioethanol; pre-treatment; raw starch.

Introduction

Throughout the 20th century, oil and its derivatives became the main energy source, thus leading to a global economic dependence [1]. Besides this, fossil fuels are responsible for the emission of greenhouse gases, contributing to global climate changes. Biomass can make a substantial contribution to supplying future energy demand in a sustainable way. It is presently the largest global contributor of renewable energy [2]. Bioethanol is currently the

most widely used liquid biofuel in the world. Global ethanol production was about 13000 million gallons in 2007, and production has almost doubled over the past years, with a production approaching 26000 million gallons for 2015 [3]. At present, bioethanol is produced exclusively via 1st generation technologies, utilizing sugar and starch-rich feedstocks, as no commercial size 2nd generation cellulosic ethanol facilities are presently in operation [4]. Starch is a natural, cheap, available, renewable, and biodegradable carbohydrate polymer produced by many plants as a source of stored energy. Bioethanol production using starch rich materials, represents a cost-effective means for the production of bio-alcohol comparing to the use of lignocelluloses [5]. Corn is the dominant material in the starch to ethanol transformation industry worldwide [6]. However , wheat is the first available material for the production of bioethanol in some regions [4]. Traditional conversion of starch into alcohol requires a two-stage process: hydrolysis of starch by acid or amylolytic enzyme and fermentation by anaerobic bacterium or yeast. Simultaneous saccharification and fermentation with mixed is an effective method for the direct fermentation of starch offering the advantages of realization in one reactor and the glucose produced is rapidly converted into ethanol (Beschkov et al.,1984). However, in this system the ethanol yield decreases because starch is consumed by the growth of amylolytic microorganisms. To increase the production of ethanol, it is necessary to breed a microorganism by a genetic manipulation, which can directly ferment starch into ethanol [7, 8]. In the present study, two starch-rich products *ie:* wheat and corn flours; were used as biomass for the production of ethanol. The raw starch contained in the flours was pre-treated with crude amylase produced by the strain *B. subtilis* TLO3, which optimal production conditions were previously investigated. Thereafter, the released sugars were fermented using the yeast *S. cerevicea.* A comparison between the two substrates was done concerning the reducing sugars obtained and the ethanol produced.

Methodology

1. Biological material

Wheat (*Triticum durum*) and corn (*Zea mays)* flours were used as starch-rich substrate for the production of bioethanol. The strain *Bacillus subtilis* TLO3 (accession number KR262718) was isolated from rhizosphere of olive tree in Tlemcen (Algeria) after a screening program from different sources based on amylase production and physiological features (data not shown). The yeast *S. cereviseae* was obtained from a commercial source.

2. Amylase production optimization

Medium composition and production conditions were optimized to obtain the best combination for optimal amylase production by the strain *B. subtilis* TLO3. The optimization was done using the OVAT (One-Variable-at-Time) method and amylase activity was analysed by estimating the released reducing ends of sugar according to the dinitrosalicylic acid (DNS) method of [9]. The experiments were realized using basal media containing 0,5% starch and 0,2% yeast extract with pH 7 and shaking at 150 rpm. The production media were sterilized by autoclaving at 121° for 20min. The flasks were then cooled and inoculated with the 24h culture seed 4%. The following paramaters were tested: Secondary carbon sources (Glucose, cellobiose, saccharose, xylose, galactose, lactose, cellulose, tween 20, tween 80, glycerol (0,5%)) ; Nitrogen sources (peptone, casein, yeast extract, Urea, gelatine (0,25%), Sodium nitrate and sodium nitrite (0,5%)); NaCl concentration (2,5 , 5, 10, 15, 20%, 25%) ; pH (5, 6 ,7, 8, 9, 10) ; Temperature (28°C, 37°C, 50°C, 60°C and 80°C) ; Inoculum size (0,5, 1, 2, 3, 4, 5%) and Incubation time (24, 48, 72 hours).

3. Amylase production

Two flasks containing 120 ml amylase production optimized medium were prepared. The strain *B. subtilis* TLO3 was cultivated on nutrient broth for 24h at 60°C. Five per cent of the culture was inoculated to the amylase production medium. After 24h of incubation at 37°C

under orbital shaking 150 rpm, the medium was centrifuged at 10000 rpm during 10 min at 4°C and the supernatant was used as crude amylase for the saccharification of the flours.

4. Wheat and corn flours saccharification

Ten grams of each flour was added to the crude supernatant then incubated under orbital shaking 150rpm at 45°C for 4h for wheat flour, and at 35°C for 24h for corn flour, in accordance with time and temperature of saccharification necessary for each starch [10, 11]. Samples were taken every hour and centrifuged at 10000 rpm for 10min to determine the amount of reducing sugars released. Media were finally centrifuged at 10000 rpm for 10 min at 4°C ; then the supernatants autoclaved at 121°C for 20 min.

5. Reducing sugars fermentation using *Saccharomyces cereviseae*

The strain *S. cereviseae* was cultivated on a Peptone-yeast-glucose medium for 48h at 30°C. Each saccharification medium was inoculated with 5% yeast culture. The media were then incubated at 30°C for 24h and samples were taken each hour for the monitoring of reducing sugar and ethanol concentrations.

6. Determination of reducing sugars and ethanol production

The amount of reducing sugars was measured before and after flours saccharification and throughout the fermentation process using the DNS method [9]. Concerning the ethanol production, it was determined by the colorimetric method described by [12]. A mixture containing 0.5ml sample to be assayed, mixed with 0.5ml sodium dichromate reagent; 0.5ml acetate buffer pH 4.3 and 2.5ml sulphuric acid 1N. The solution was then vortexed for 1min then incubated at room temperature for 120min. The absorbance was read at 578nm using a spectrophotometer and a standard curve was plotted using different ethanol concentrations.

Results and discussion

1. Amylase production optimization

The highest amylase production was obtained using 0.5% starch as essential carbon source, 0.5% xylose as secondary carbon source, 0.25% urea as nitrogen source, 2.5% NaCl and 3% inoculum size. The production was optimum at initial pH: 7, temperature 50°C and 24 h incubation period at 150 rpm shaking. The high production yield noted at high temperature (50°C), pH range from 6 to 9 and in short time (24h), are promising results for application at large scale allowing high amylase production and consequently elevated concentrations of fermentable sugars for bioethanol production.

2. Wheat and corn flours amylase pre-treatment

Flours starch saccharification was performed using crude amylase produced by *B. subtilis* TLO3 (Figure 1, Figure 2). A good yield of released reducing sugars was noted for both flours. Thus, a percentage of 70% and 91% of reducing sugars was obtained during the saccharification of wheat and corn flours, respectively; proving the efficiency of starch saccharification of the crude amylase produced by *B. subtilis* TLO3. Several studies reported raw starch saccharification for bioethanol production using amylase produced by *Bacillus spp*. strains [13, 14].

3. Reducing sugars fermentation and ethanol production

The monitoring during 24h of reducing sugars fermented and ethanol produced is shown in Figure 3 and Figure 4. The choice of duration of 24h for the fermentation was motivated by the advantage of production of ethanol in a short time which allows doing considerable energy savings. The reducing sugars concentration at the beginning of the fermentation was 142 µg/ml and 214 µg/ml, for wheat and corn flours, respectively. This difference could be due to the starch content of corn 79% [15], which is superior to that of wheat 62% [16]. The presence of resistant starch inaccessible to amylase enzymes up to 13% for wheat flour and 8.1% for corn flour [17], can also explain that difference. The monitoring of reducing sugars concentration during the fermentation showed a slight increase in the 3 first hours, which can

be explained by a secretion of amylase by the yeast *S. cereviseae.* This was followed by a continuous decrease reaching 42% and 79% less for wheat flour and maize, respectively, comparing to initial concentrations. This decrease indicates clearly that the yeast transformed the reducing sugars, glucose in particular, obtained after the saccharification of the flours starch. Concerning ethanol production, the monitoring showed a production yield of 0.92 g/l (2%) for the wheat flour and 1.1 g/l (2.4%) for the corn flour after 24h. For the wheat flour the production was steady during the 4 first hours, then a continuous increase was noticed from the fifth hour. For the corn flour, after an increase during the 3 first hours, the amount of ethanol declined during 3 hours, then resumed the increase in a continuous manner until 24h. This decrease could be due to a contamination by an acetic acid bacteria, which could ferment ethanol and transform it to acetic acid by and oxydo-reduction reaction [18], which represents a limiting factor in bioethanol production process. The best ethanol yield was obtained using corn flour because of the higher starch content, and thus fermentable sugars. Evaluative studies concerning starch for ethanol yield optimization described five criteria that influences the functional properties of starch : amylose/ amylopectin content [19-23], the morphology of starch granule [24], the fine structure of amylopectin [25-27], thermal properties [20, 22] and pasting properties [22].

Conclusion:

Bioethanol production using starch rich substrates, in particular corn, represents a cost-effective means for the production of bio-alcohol comparing to the use of lignocelluloses. Amylase pre-treatment of starchy materials gave encouraging results in ethanol production, especially for corn flour. The flours composition affects the fermentable sugars yields and thus the ethanol production. The prior optimization of amylase production conditions is an essential step for an optimal hydrolysis of starch. The bioethanol production conditions could be optimized to achieve a successful scale-up to industrial level production.

Disclaimer: - The title and abstract of this manuscript was presented in the conference Conference Name: “The Energy and Materials Research Conference - EMR2017, At Lisbon (Portugal)” available link is “https://www.researchgate.net/publication/316545287_Production_of_raw_starch_degrading_amylase_by_Bacillus_subtilis_TLO3_and_its_application_in_bioethanol_production_from_starch-rich_flours” Date- April 2017

References

1. Cinelli, B.A., et al., *A brief review on the emerging technology of ethanol production by cold hydrolysis of raw starch.* Fuel, 2015. **150**: p. 721-729.
2. IEA_Bioenergy. *Bioenergy – a sustainable and reliable energy source. A review of status and prospects*. 2009 [cited 2016 December 2016]; ExCo: 2009:06:[Available from: http://www.ieabioenergy.com/publications/main-report-bioenergy-a-sustainable-and-reliable-energy-source-a-review-of-status-and-prospects/.
3. Renewable Fuel Association. *World Fuel Ethanol Production* 2015 [cited 2016 December 12]; Available from: http://ethanolrfa.org/resources/industry/statistics/.
4. Saunders, J., D.B. Levin, and M. Izydorczyk, *Limitations and challenges for wheat-based bioethanol production*, in *Economic Effects of Biofuel Production*, M.A. Dos Santos Bernardes, Editor. 2011, InTech: Rijeka. p. 430-452.
5. McAloon, A., et al., *Determining the cost of producing ethanol from corn starch and lignocellulosic feedstocks.* National Renewable Energy Laboratory Report, 2000.
6. Gnansounou, E. and A. Dauriat, *Ethanol fuel from biomass: A review.* Journal of Scientific and Industrial Research, 2005. **64**(11): p. 809.

7. Nakamura, Y., et al., *Alcohol fermentation of starch by a genetic recombinant yeast having glucoamylase activity.* Biotechnology and bioengineering, 1997. **53**(1): p. 21-25.

8. Shigechi, H., et al., *Direct production of ethanol from raw corn starch via fermentation by use of a novel surface-engineered yeast strain codisplaying glucoamylase and α-amylase.* Applied and Environmental Microbiology, 2004. **70**(8): p. 5037-5040.

9. Miller, G.L., *Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar.* Analytical Chemistry, 1959. **31**(3): p. 426-428.

10. Textor, S.D., et al., *Cold enzyme hydrolysis of wheat starch granules.* The Canadian Journal of Chemical Engineering, 1998. **76**(1): p. 87-93.

11. Uthumporn, U., I.S.M. Zaidul, and A.A. Karim, *Hydrolysis of granular starch at sub-gelatinization temperature using a mixture of amylolytic enzymes.* Food and Bioproducts Processing, 2010. **88**(1): p. 47-54.

12. Sumbhate, S.V., et al., *Colorimetric Method for the Estimation of Ethanol in Alcoholic-Drinks.* 2012, 2012. **1**(1): p. 6.

13. Somda, M., et al., *Improvement of bioethanol production using amylasic properties from Bacillus licheniformis and yeasts strains fermentation for biomass valorization.* Asian J. Biotechnol, 2011. **3**: p. 254-261.

14. Tran, H.T.M., et al., *Potential use of Bacillus subtilis in a co-culture with Clostridium butylicum for acetone–butanol–ethanol production from cassava starch.* Biochemical Engineering Journal, 2010. **48**(2): p. 260-267.

15. Jaekel, L.Z., et al., *Influence of xylanase addition on the characteristics of loaf bread prepared with white flour or whole grain wheat flour.* Food Science and Technology (Campinas), 2012. **32**: p. 844-849.

16. Neves, M.A.d., et al., *Production of alcohol by simultaneous saccharification and fermentation of low-grade wheat flour.* Brazilian Archives of Biology and Technology, 2006. **49**: p. 481-490.

17. Murphy, M.M., J.S. Douglass, and A. Birkett, *Resistant starch intakes in the United States.* Journal of the American Dietetic Association, 2008. **108**(1): p. 67-78.

18. Gómez-Manzo, S., et al., *The Oxidative Fermentation of Ethanol in Gluconacetobacter diazotrophicus Is a Two-Step Pathway Catalyzed by a Single Enzyme: Alcohol-Aldehyde Dehydrogenase (ADHa).* International Journal of Molecular Sciences, 2015. **16**(1): p. 1293-1311.

19. Lee, M.-R., B.G. Swanson, and B.-K. Baik, *Influence of amylose content on properties of wheat starch and breadmaking quality of starch and gluten blends.* Cereal chemistry, 2001. **78**(6): p. 701-706.

20. Wu, X., et al., *Factors impacting ethanol production from grain sorghum in the dry-grind process 1.* Cereal Chemistry, 2007. **84**(2): p. 130-136.

21. Wu, X., et al., *Effects of amylose, corn protein, and corn fiber contents on production of ethanol from starch-rich media 1.* Cereal chemistry, 2006. **83**(5): p. 569-575.

22. Zhao, R., et al., *Comparison of waxy vs. nonwaxy wheats in fuel ethanol fermentation.* Cereal Chemistry, 2009. **86**(2): p. 145-156.

23. Zhu, L.-J., et al., *Characterization of arsenic-resistant endophytic bacteria from hyperaccumulators Pteris vittata and Pteris multifida.* Chemosphere, 2014. **113**: p. 9-16.

24. Liu, Q., et al., *Investigation of digestibility in vitro and physicochemical properties of A-and B-type starch from soft and hard wheat flour.* Cereal Chemistry, 2007. **84**(1): p. 15-21.

25. Ao, Z. and J.-l. Jane, *Characterization and modeling of the A-and B-granule starches of wheat, triticale, and barley.* Carbohydrate Polymers, 2007. **67**(1): p. 46-55.

26. Sasaki, T., et al., *Comparison of physical properties of wheat starch gels with different amylose content.* Cereal chemistry, 2002. **79**(6): p. 861-866.

27. Zhang, G., M. Sofyan, and B.R. Hamaker, *Slowly digestible state of starch: mechanism of slow digestion property of gelatinized maize starch.* Journal of agricultural and food chemistry, 2008. **56**(12): p. 4695-4702.

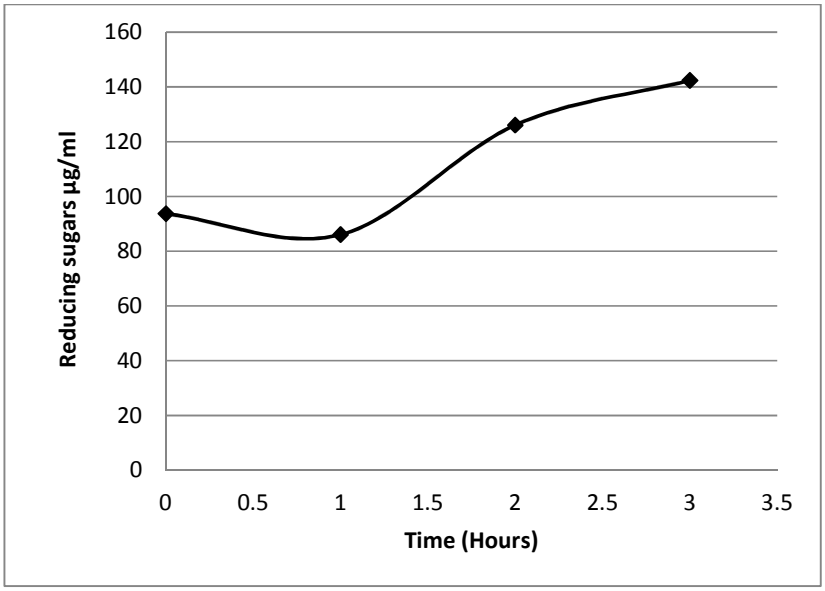
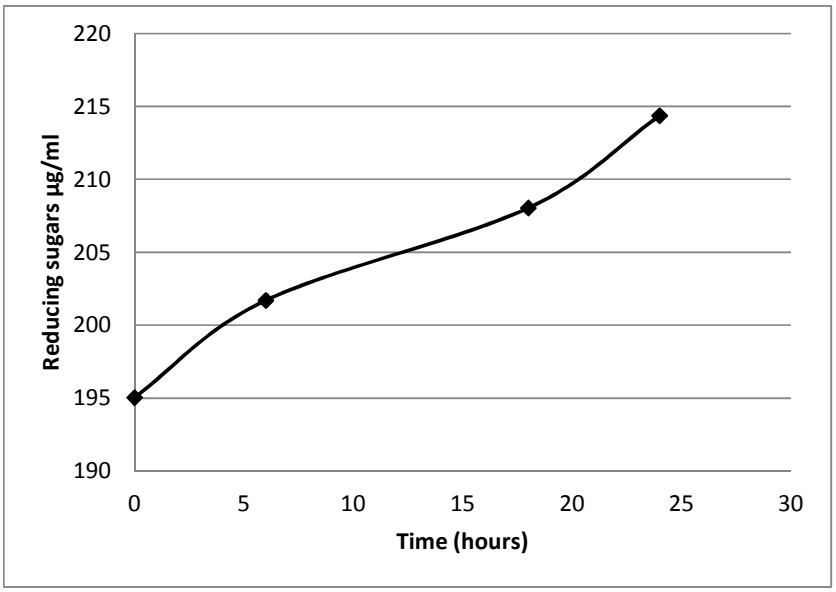


Figure 1. Reducing sugars released during the saccharification of wheat flour.

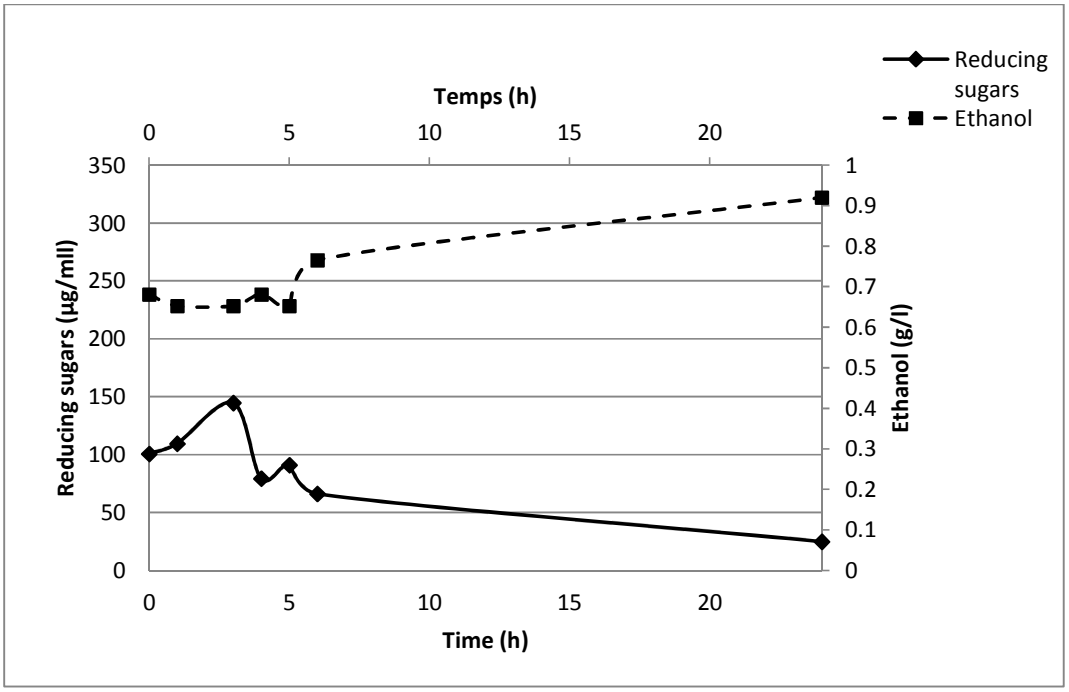
240



241

242 **Figure 2. Reducing sugars released during the saccharification of corn flour.**

243



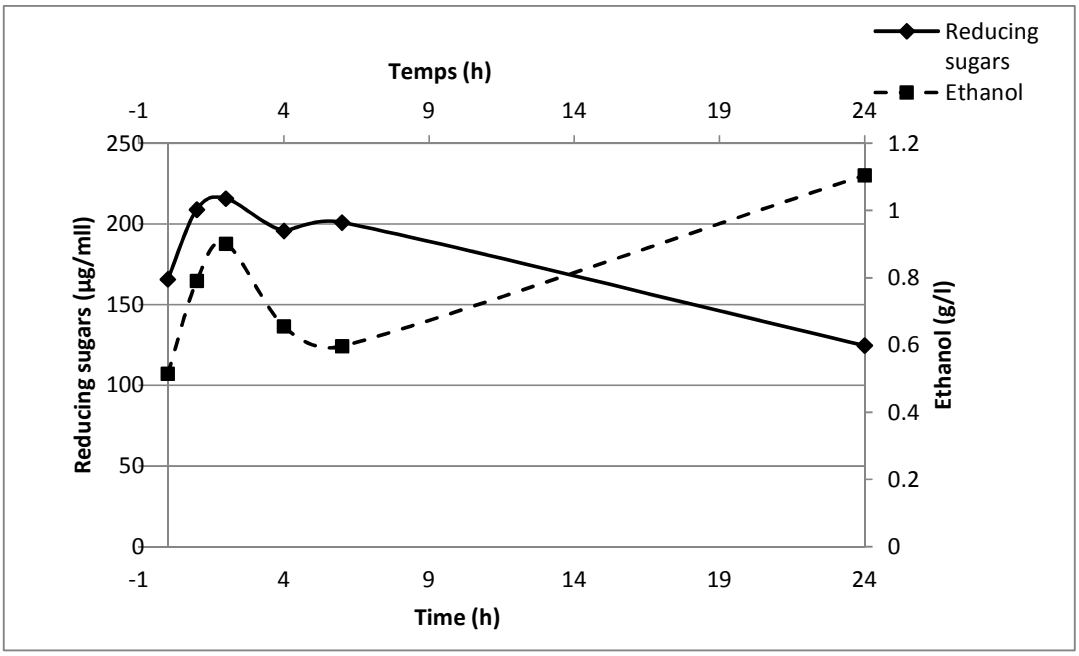
244

245 **Figure 3. Monitoring of ethanol production and reducing sugars during the fermentation of wheat flour using *Saccharomyces cereviseae.***

246

247

248



249

250 **Figure 4. Monitoring of ethanol production and reducing sugars during the fermentation of corn flour using *Saccharomyces cereviseae*.**