Effect of*Bacillus subtilis* TLO3 amylase pre-treatment on ethanol production from raw starches

Abstract

Bioethanol is currently the most widely used liquid biofuel in the world. Starch rich crops occupy the first place as biomass for bioethanol production.Amylases(EC 3.2.1.1) are enzymes that hydrolyses starch into sugar units, and pre-treating starch with amylolytic bacteria or directly by amylase might have a positive effect on fermentable sugars concentrations and ultimately result in increased ethanol yields.

In this study, an amylase producer strain *Bacillus subtilis* TLO3 newly isolated from rhizospheric soil was used for amylase production; after investigating the best combination of physico-chemical parameters. The crude enzyme was used for the pre-treatment of raw corn and wheat starches. Immediately afterwards, the yeast *Saccharomyces cerevisiae*was inoculated into the saccharified starch solutions for fermentation. Measures were done for total reducing sugars and ethanol production all along the fermentation process.

Thus, the best amylase production was obtained using 0.5% starch; 0.5% xylose; 0.25% urea; 2.5% NaCl; 3% bacterial inoculum; pH 7; temperature 50°C and 24h incubation time. Amounts of reducing sugars of 70% and 91% were obtained after saccharification of wheat and corn starch, respectively, by crudeamylase. 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; optimization; *Bacillus subtilis* TLO3; bioethanol; pre-treatment; raw starch; *Saccharomyces cerevisiae*.

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 a majorcontributorto greenhouse gases emissions, leading 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]. Bioethanol can be produced using different biomass, but at present it 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: hydrolysisof starch by acid or amylolytic enzyme and fermentation by anaerobic bacterium or yeast. Simultaneous saccharification and fermentation with mixed cultures 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 [7]. 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[8].In the present study, two starch-rich products (wheat and corn flours); were used as substrates 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 in solution were fermented using the yeast*S. cereviseae.* The results obtained for the two flours were compared to determine the effect of amylase pre-treatment on each substrate concerning starch hydrolysis and thus ethanol production.

Methodology

1. Biological material

Wheat (*Triticum durum*) and corn (*Zea mays)* flours were used as starch-rich substrates for the production of bioethanol.The strain *Bacillus subtilis* TLO3 (accession number KR262718) was isolatedaseptically(15 cm depth) from rhizospheric soil of olive tree in the region of Tlemcen (Algeria)and selected after a screening program from different sources based on amylase production and physiological features (data not shown). The strain*S. cereviseae*S288Cwas 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 One-Variable-at-Time(OVAT) method and amylase activity was analysed by estimating the released reducing ends of sugar according to the dinitrosalicylic acid (DNS) method ofMiller[9]. The sample to be assayed was mixed with starch 1% (v/v)buffered in sodium phosphate pH 6.8; then the mixture was incubated for 30 min at 50°C. The reaction was stopped by adding the same volume of DNS reagent and boiled for 10 min at 100°C. The absorbance was read using a spectrophotometer at 540nm.

The experiments were realized using basal media containing 5g potato starch and 2g yeast extract per 1000 ml distilled water (w/v), with pH 7 and shaking at 150 rpm. The production media were sterilized by autoclaving at 121°C for 20min. The flasks were then cooled and inoculated with the 4% (v/v) *B. subtilis* TLO3culture seed (DO_{600} = 0.05).

The following parameters were tested:secondary carbon sources (glucose, cellobiose, sucrose, xylose, galactose, lactose, cellulose, tween 20, tween 80, glycerol (0,5%)(w/v));nitrogen sources (peptone, casein, yeast extract, urea, gelatine (0,25%)(w/v),sodium nitrate and sodium nitrite (0,5%)(w/v)); NaCl concentration(2,5 , 5, 10, 15, 20,25% (w/v)) ; 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% (v/v)) andincubation time (24, 48, 72 hours).

3. Amylase production

Two 500 ml flasks containing 120 ml amylase production optimized medium were prepared. The strain *B. subtilis* TLO3was cultivated on nutrient broth for 24h at 50°C. Threeper cent of the culture (v/v) was inoculated to the amylase production media. After 24h of incubation at 50°C under orbital shaking 150 rpm, the mediawere centrifuged at 10000 rpm during 10 min at 4°C and the supernatantswere 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* S288C was cultivated on a Peptone-yeast-glucose PYG medium containing 1.25g peptone; 1.25g yeast extract and 3g glucose per 1000ml of distilled water (w/v); for 48h at 30°C. Each saccharification medium was inoculated with 5% yeast culture(v/v) (DO_{600}= 0.05). 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 Sumbhate *et al.*[12]. A mixture containing 0.5ml sample to be assayed, was 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 (367 ± 6 U/ml) was obtained using 0.5% starch as essential carbon source, 0.5%(w/v)xylose as secondary carbon source, 0.25%(w/v)urea as nitrogen source, 2.5%(w/v)NaCl and 3%(v/v)inoculum size. The production was at its optimumat initial pH 7, temperature 50°C and 24 h incubation period at 150 rpm shaking.

Many Firmicutes bacteria are able to utilize xylose as carbon source (Gu et al., 2010). Xylose may be implied in ribose synthesis, an important sugar in nucleic acid formation. Indeed, Park *et al.*[13]reported the isolation of transketolase deficient *B. subtilis* strain, which was able to produce D-ribose from xylose. Nahas and Waldemarin[14] showed that xylose was

among the best supplementary carbon sources for highest amylase production using the fungi *Aspergillusochraceus*.

Among organic and inorganic nitrogen sources employed, urea showed the highest amylase activity, followed by sodium nitrate. This shows that this strain has no preference between inorganic and organic nitrogen source for amylase production. Nagarajan *et al.,* [15]reported maximum amylase production by *B.subtilis* strain using urea as nitrogen source.

The high production yield noted at high temperature is an asset in industrial enzyme production because it influences both bacterial growth and amylase production[16].Many studies reported optimum amylase production in this temperature range using *Bacillus* strains [17, 18, 19].

Also, maximum amylase production in short time(24h), represent promising results for application at large scale allowing considerable energy savings.Similar works reported maximum amylase activity after 24h using Bacillus strains [20, 21].Optimization results are presented in Table 1.

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, percentages of 70% and 91% of reducing sugars were 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[22, 23, 24, 25].

3. Fermentation of reducing sugars 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 an incubation time 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 100 µg/ml and 165 µg/ml, for wheat and corn flours, respectively. This difference could be due to the starch content of corn 79% [26], which is superior to that of wheat 62% [27]. The presence of resistant starch inaccessible to amylase enzymes up to 13% for wheat flour and 8.1% for corn flour [28], 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. Indeed, thestrain *S. cereviseae*S288c possesses anα-glucosidase MAL32expressed in early log phase[29].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 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, and 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[30, 31, 32], 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 [33, 34, 35, 36, 37], the morphology of starch granule [38], the fine structure of amylopectin [39, 40, 41], thermal properties [34, 36] and pasting properties [36].

Conclusion:

Bioethanol production using starch rich substrates remains, to the present, the most cost-effective means for bio-alcohol production; due to ease of saccharification comparing to lignocelluloses. Amylase production optimization has indicated that *B.subtilis* TLO3 is a promising candidate for starch transformation industrydue to high amylase activity, production at high temperature and reduced time. Raw corn and wheat starches were pre-treated with crude amylase produced using the obtained parameters combination and high saccharification yields were obtained.Also good ethanol productionwasachieved, after fermentation of the released reducing sugars by the yeast *S. cereviseae* S288C.

Corn flour showed the best saccharification yield and ethanol production, confirming that it is, so far, the best starch substrate for ethanol production. For further improvement, statistical design optimization ofbioethanol production conditions is envisaged, with the aim to achieve a successful scale-up to industrial level production.

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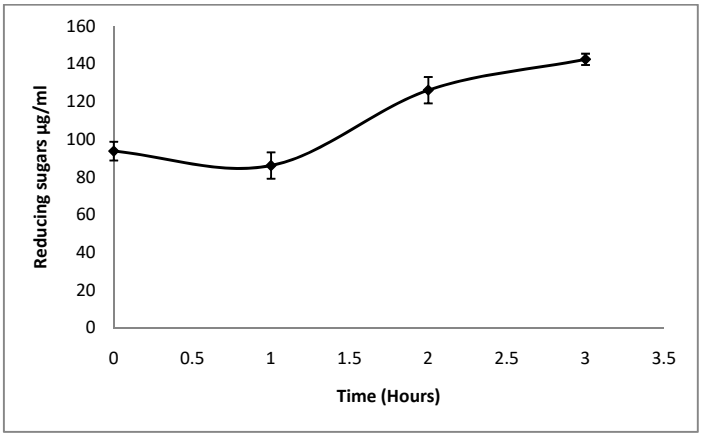
Table 1 : Results of amylase production optimization

Secondary carbon source	Amylase activity (U/ml) (mean ± SD)
Glucose	182.5 ± 3
Galactose	254.44 ± 7
Xylose	347.22 ± 1
Cellobiose	231.66 ± 1
Saccharose	118.88 ± 2
Lactose	297.22 ± 8
Maltose	244.16 ± 6
Cellulose	81.66 ± 5
Glycerol	159.72 ± 1
Tween 20	133.33 ± 7
Tween 80	117.5 ± 5
Nitrogen sources	**Amylase activity (U/ml)**
Peptone	86,66666667 ± 2
Yeast ext	126,6666667 ± 5
Casein	134,1666667 ± 7
Urea	165,2777778 ± 7
Gelatin	141,6666667 ± 6
NaNo2	61,11111111 ± 3
NaNo3	153,3333333 ± 5
NaCl (%)	**Amylase activity (U/ml)**
0	108,6111111 ± 1
2,5	151,9444444 ± 5
5	126,6666667 ± 10
10	94,44444444 ± 5
15	83,33333333 ± 3
20	63,88888889 ± 5
25	55 ± 2
pH	**Amylase activity (U/ml)**
5	109,7222222 ± 5
6	112,5 ± 7
7	153,8888889 ± 8
8	131,3888889 ± 8
9	108,3333333 ± 5
10	100,5555556 ± 2
Temperature	**Amylase activity (U/ml)**
28	93,88888889 ± 1
37	164,7222222 ± 4
50	167,2222222 ± 8
60	194,4444444 ± 5
80	45,27777778 ± 3
Inoculum size (%)	**Amylase activity (U/ml)**
0,5	115 ± 3
1	101,3888889 ± 3
2	107,5 ± 5
3	113,3333333 ± 7
4	108,6111111 ± 6
5	103,8888889 ± 1
Incubation time (h)	**Amylase activity (U/ml)**
24	108,6111111 ± 1
48	95,27777778 ± 5
72	85,83333333 ± 3



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

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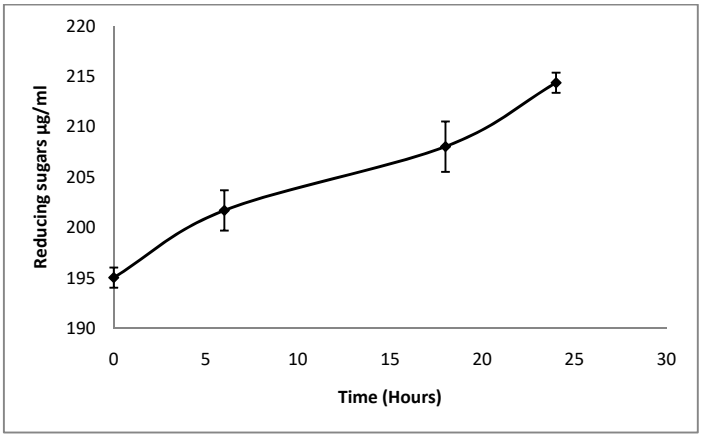

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353 **Figure 2. Reducing sugars released during the saccharification of corn flour.**

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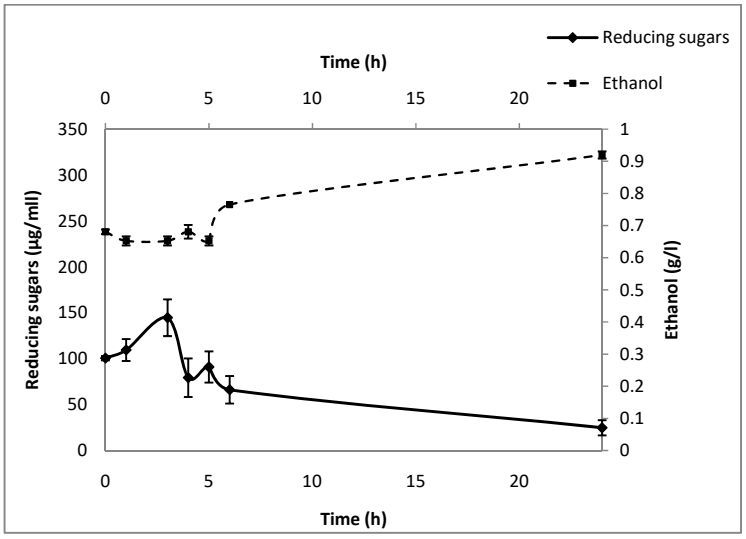

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Figure 3. Amounts of ethanol produced and reducing sugars fermented during the

356 **fermentation of wheat flour using *Saccharomyces cereviseae.***

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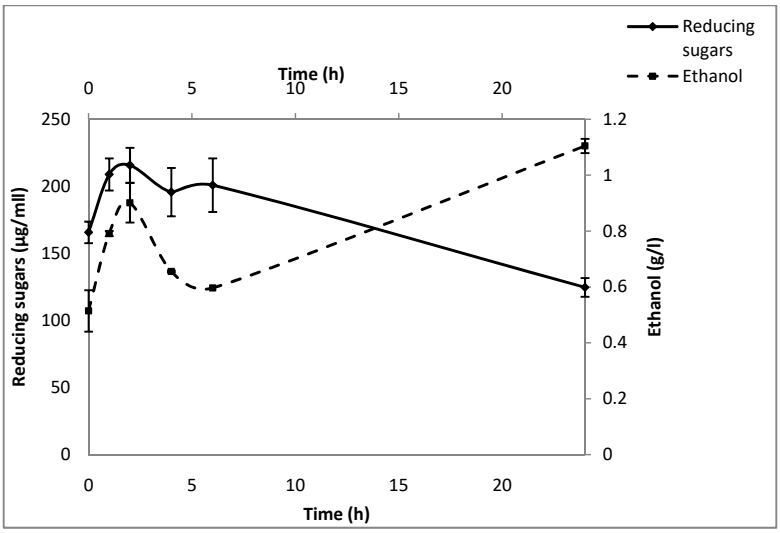

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359 **Figure 4. Amounts of ethanol produced and reducing sugars fermented during the fermentation of corn flour using *Saccharomyces cereviseae.***