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PRODUCTION OF BIOETHANOL FROM CARROT DISCARDS

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ABSTRACT:

A revalorization of discarded carrots as substrate for the production of second-generation ethanol is proposed. In order to increase the fermentable sugar concentration of the musts two strategies were studied: Strategy 1 consisted in the enzymatic hydrolysis of bagasse must and Strategy 2 by which carrots were milled, dropped into distilled water and hydrolyzed with different enzymes prior to compressing and filtering to obtain carrot must. By applying Strategy 2 using 0.05 % (v/v) of the enzyme Optimase CX255 at 70 °C and pH 5.5 during 2.5 h, the fermentable sugars extracted increased 3.5 times. In this way, the production of 77.5 L of ethanol for each ton of discarded carrots was achieved. This process yielded bagasse as byproduct, which could be used for animal feed.

KEYWORDS: carrot, fermentation, ethanol, enzymatic hydrolysis

ABBREVIATIONS:

RS: reducing sugars

TS: total sugars

CM: carrot must

BM: bagasse must

DC: discarded carrot

1. INTRODUCTION

At present, the dependence on fossil fuels is still strong (85–90 %) in spite of their rapid depletion. In this context, the search for alternative energy sources has become a crucial issue that leads to the development of better technologies for second-generation biofuel production (Simsa et al., 2010).

The use of solar energy by means of the photosynthesis which produces biomass (Kreuger et al., 2011) is the most important source of renewable raw materials (Clark, 2005; Sánchez and Cardona, 2008). The acid or enzymatic hydrolysis of polysaccharides such as cellulose, hemicellulose, starch, inulin and chitosan in hexoses and pentoses allows their use in the alcoholic fermentative process. Of both types of hydrolysis, the enzymatic one allows obtaining a pure product with a low energy demand and a minor effluent production (Tomás, 2009; Ovando and Waliszewski, 2005) but the yield differs considerably depending on the applied technology (Sun and Cheng, 2002). In this way, bioethanol can be obtained from energy crops and lignocellulosic residues. The sustainability of the biotransformation processes must be analyzed from an economic and environmental point of view. Besides the high cost of current technologies and enzymes, another economic issue is to select cheap and abundant raw materials. That is why the use of regional agricultural and agro-industrial discards is very attractive to produce second-generation bioethanol (Laufenberg et al., 2003; Sánchez and Cardona, 2008; Aimaretti, 2011).

In Santa Fe (Argentina), a particular case is carrot (*Daucus carota*) cultivation whose average yield is nearly 40 tons (t) ha^{-1} with a cultivated area of

approximately 1,500 ha. During the harvest time, 20-100 t of carrots with an optimal degree of freshness and maturity are discarded daily due to a sizing problem and then directed to animal feed (Aimaretti, 2006). Carrot is one of the most efficient crops in biomass accumulation (Diamantopoulou et al., 2011) and it is one of the few plants that accumulate free sugars into vacuoles (40-60% of total carbohydrates) as reserve carbohydrates. 95% of free sugars are composed of sucrose, fructose and glucose, and reducing sugars (RS) (fructose and glucose) are present in an equimolecular amount. The ratio sucrose/RS increases while the plant reaches maturity. After harvesting and during the cold storage, the ratio begins to fall down (Simon, 2000; Suojala, 2000).

Taking into account the above considerations, the present study had two main objectives: i) to evaluate the production of second-generation bioethanol using discarded carrots as raw material, and ii) to enhance the ethanol yield of the process by enzymatic hydrolysis in order to increase the fermentable sugar concentration of the must.

2. MATERIALS AND METHODS

2.1. Raw material, handling and storage

Discarded roots of carrot (DC) (*Daucus carota*) were collected in November-December 2010 from a packing shed in the Santa Fe area (31°25'S, 60°20'W), Argentina. These discards were by-products from carrot packing processes, in which plant leaves were cut and whole roots were washed, dried and selected before packing. DC were stored in shed under ambient conditions (20-32 ˚C; 50-60

% humidity). During delivery of random representative samples obtained by sampling methods (ANMAT, 2010), carrots were packed in a polypropylene container and moved to the laboratory where they were used immediately.

2.2. Primary carrot processing

For must preparation, DC were processed after discarding those rotten sections, to extract their juice by a continuous milling, compressing and filtering treatment. As a result of the treatment, two fractions were obtained: i) carrot juice, which was called carrot must (CM) and ii) carrot bagasse which was utilized for the preparation of bagasse must (BM). The yield of the process was: 0.54 Kg kg^{-1} of CM and 0.46 kg kg^{-1} of bagasse. On the other hand, BM was prepared by dipping bagasse into a water volume in a proportion of 0.35 kg L^{-1}.

2.3. Enzymes

The enzymes used in the hydrolysis reactions and the operational conditions performed are described in Table 1. The pretreatments were performed in a stirred tank reactor manufactured at UNL, Argentina, equipped with a controlled heating system. Agitation speed was regulated at 20 rpm for all the experiments. Each enzyme has an indicated value of pH defined by its producers, which was adjusted with diluted sulfuric acid.

(Table 1, here)

2.4. Enzymatic hydrolysis of Bagasse Must

The seven commercial enzymes (Table 1) were evaluated under the conditions recommended by suppliers using BM as substrate. In each experiment, the enzyme dose was added after adjusting initial pH and temperature. The

hydrolysis time was 2.5 h and homogeneous samples were taken every 30 min. The samples were centrifuged during 10 min at 5,000 x g and the supernatants were used for determining the concentration of total sugars (TS) and reducing sugars (RS) by using the DNS method (see 2.8.2) and to analyze the carbohydrate composition (see 2.8.3) before the fermentation assay. All experiments were performed in duplicate and technical repeats were performed during each single experiment.

2.5. Enzymatic hydrolysis of carrot prior to prepare carrot must

Depending on the enzymatic activity and their availability, enzymes 1, 2, 4 and 5 (Table 1) were tested for enzymatic hydrolysis prior to preparing CM. In this way, DC was milled to a particle size minor to 4 mm, dropped into distilled water and then enzymatically hydrolyzed. For these assays, batches containing 0.5 Kg of milled DC dipped into water in a total volume of 1 L were mixed with the enzymes, after adjusting the initial pH and temperature. The hydrolysis was performed during 2.5 h and homogeneous samples were taken every 30 min of reaction. The samples were compressed and filtered to obtain CM. The concentration of RS and TS was determined using the DNS method (see 2.8.2). All experiments were performed in duplicate and technical repeats were performed during each single experiment.

2.6. Microorganism

Saccharomyces cerevisiae CCUB filtered and discarded by a local brewing industry after five operative cycles, was utilized as biocatalyst in the fermentation reactions. Whole yeast cells were kept in a sterile container, without nutrient

addition at 4 ºC and saturation humidity during four days (Aimaretti and Ybalo, 2012). For the inoculum preparation, cells were suspended in an isotonic solution and counted by a differential direct count method in order to calculate the volume required (Alfenore et al., 2002).

2.7. Fermentation conditions

For alcoholic fermentations, fresh must was used in every case and its pH was adjusted to 4.5. The inoculum was adjusted to a value of 10^8 cell mL^{-1} (Aimaretti and Ybalo, 2012). Batch fermentations were developed to 28 °C, in a 500 mL stirred tank bio-reactor (self-manufactured), equipped with a controlled heating system. The agitation speed was regulated at 100 rpm in all the experiments. A reactor hold-up of 0.25 was used, according to Laopaiboon et al. (2009). The alcoholic fermentation progress was monitored following CO_2 production, which was collected in a gasometric probe (ANMAT, 2010). Samples at different reaction times were taken and submitted to centrifugation. Supernatants were transferred to Eppendorf tubes (1.5 mL of capacity) for their storage and conservation at – 20°C. All experiments were performed in triplicate.

2.8. Analytical methods

2.8.1. Moisture

Moisture was determined using Approved Method 44-15A (AACC, 2005).

2.8.2. Sugar concentration

The concentration of reducing sugars was measured by the 3.5-dinitrosalicylic acid (DNS) method (Aimaretti and Ybalo, 2012). The total sugar was

assayed by the same method after acid hydrolysis (1.2 mol L^{-1} HCl, at 65 °C for 15 min), neutralization with 1 mol L^{-1} NaOH and filtration (Yu et al., 2009).

2.8.3. Carbohydrate composition

The analysis of carbohydrate composition in hydrolyzed musts was performed by high-performance anion exchange chromatography with pulsed amperometric detection HPAEC-PAD using an ICS-300 system (Dionex Corp, Sunnyvale, CA, USA) with a CarboPac™ PA100 column (4 x 250 mm, Dionex Corporation, CA, USA) with a guard column (4 x 50 mm). The eluent and flow rate were 16 mM NaOH and 1 mL min^{-1} at 25 °C, respectively. Before the HPAEC-PAD analysis, the hydrolyzed samples were heated up to 85 °C for 5 min in order to inactivate the enzymes; then, they were filtered and treated in a separate Sephadex G-25 column (Pharmacia, PD10) to remove high molecular weight molecules that can affect the HPLC column. 5 μL of hydrolyzed sample together with 5 μL internal standard (Lactulose, Anedra > 99.5 %) were injected in the chromatographer (Aimaretti, 2011). Measurements were made in duplicate.

2.8.4. Ethanol concentration

Ethanol concentration was determined by GC (PERKIN-ELMER, Sigma 3B, Dual FID Chromatograph, United States). A FID detector and a packed column of Chromosorb 102 (2.0m length) were employed. The column oven was operated isothermally at 150 °C and the injection and detector ports were kept at 195 ºC and 220 ºC, respectively. Nitrogen was used as carrier gas with a flow rate of 30 mL min^{-1} and the combustion gas was a mixture of hydrogen and air. Isopropanol

(Anedra, >99.9%) was used as internal standard (Ratnam et al., 2003). In the chromatograph of every fermented sample, only two peaks appeared, one corresponding to the internal standard, and the other to ethanol.

2.9. Fermentation parameters

The following fermentation parameters were calculated to compare the responses of different assays:

$Y_{p/s}$: ethanol yield per substrate was considered as the ratio of total ethanol produced and the consumed sugars, [g g^{-1}] (Colin and Bjorn, 2002).

$Y_{p/c}$: ethanol yield per carrot was considered as the ratio of total ethanol produced and used carrot (dry base), [g g^{-1}] (Colin and Bjorn, 2002).

Productivity: it was defined as total alcohol production over the total fermentation time, [g L^{-1} h^{-1}] (Colin and Bjorn, 2002).

3. RESULTS AND DISCUSSION

3.1. Carrot fermentation

In the first experiment, two different musts were obtained from the primary processing of carrots (CM and BM) and the concentration of sugars was determined. They were fermented separately under conditions indicated in Section 2.7, comparing with un-inoculated CM and BM.

The concentration of reducing sugars of CM was in average 49.8 ± 13.4 g L^{-1}, on a total of sugars 94.0 ± 11.7 g L^{-1}. After the CM fermentation, the ethanol concentration obtained was 37.1 g L^{-1}. On the other hand, the average concentration of reducing sugars of BM was 5.8 ± 0.4 g L^{-1}, the concentration of

total sugars: 29.6 ± 4.7 g L^{-1}, and the concentration of ethanol obtained by fermentation: 7.9 g L^{-1}. Meanwhile, in the control experiments in which CM and BM were not inoculated, the ethanol concentration obtained was 0.0 g L^{-1}. These results are indicative of the fact that ethanol is the main product of the metabolic way and its final yield depends on the sugar concentration, and they are in agreement with the results reported by Aimaretti and Ybalo (2012).

Taking into account that the humidity of CM was 85 % and 60 % for BM, then it can be observed that the fermentation allowed obtaining $Y_{p/c}$ values of 0.134 and 0.068 g g^{-1} after the of CM and BM, respectively. So, the total $Y_{p/c}$ of the primary carrot processing was 0.201 g g^{-1}. In brief, 38.8 L of bioethanol were obtained from 1 t of DC.

With the objective of increasing total sugar concentration of musts and the ethanol yield, two different enzymatic hydrolysis strategies were proposed for carrot processing.

3.2. Strategy 1: Enzymatic hydrolysis of bagasse must (BM).

The use of enzymes to hydrolyze the bagasse which resulted from the primary carrot processing could allow its use as a substrate for a fermentation process, thus increasing the overall productivity of DC (see 2.4).

Enzymes must be adsorbed on the particles surface of insoluble cellulose before the hydrolysis reaction begins. Thus, the three-dimensional structures of enzymes, in combination with their size and form, determine if the β-glycosidic bonds may be accessible to enzymatic attack or not (Tomás, 2009). Figure 1 shows the evolution of the reducing (Figure 1-A) and total sugars (Figure 1-B) as a function of

hydrolysis time for the different enzymes. It can be observed that all the enzymes were capable of hydrolyzing the bagasse must increasing the sugar content in different proportions. In particular, the major improvements in total sugar content of must were 78.5 % for enzyme Optimase CX255L and 75.5% for Enzigrex. It is also worth noting that the most active enzyme (OptimaseCX255L) is especially recommended by the supplier for reducing the viscosity of the components of the plant cell wall. The enzymatic hydrolysis of bagasse was also proposed as an interesting way for re-evaluating the large bagasse surplus from the juice industry (Yoon et al., 2005).

On the other hand, a noticeable effect was observed: an important increase of the reducing sugars catalyzed by Fungamyl (enzyme 7). This result is contradictory because, according to the supplier Fugamyl only presents α-amylase activity (Table 1) and only 1% of the bagasse fiber is composed of starch. This could be explained by the fact that Fungamyl presents some invertase or alpha-glucosidase residual activity to hydrolyze sucrose (Yoon et al., 2005).

(Figure 1, here)

3.2.1. Carbohydrate composition of hydrolyzed bagasse musts

The analysis of the hydrolyzed BM carbohydrate composition was carried out in order to determine the extension of the hydrolysis reaction with each enzyme. From the analysis many issues can be raised:

(i) during the filtering step of samples on Sephadex G-25 column, no high molecular weight polymers were found, which would indicate the absence of intermediate hydrolysis compounds.

(ii) during the cromatography analysis both the not-hydrolyzed BM sample and all the hydrolyzed samples presented only three peaks, corresponding to glucose, fructose and sucrose. It represents one of the advantages of the method, since all these sugars are capable of being fermented (Sun and Cheng, 2002).

The absence of degradation compounds such as xylose and/or arabinose indicated that hydrolysis process was not effective to degrade cellulose and hemicellulose. Probably the enzymatic degradation of cellulose and hemicellulose into simple sugars requires longer reaction times (Sánchez and Cardona, 2008). Therefore, the increase of TS in the hydrolyzed musts might be related with the release of the free sugars accumulated in the carrot vacuoles. Enzymatic hydrolysis was an effective way to cause destabilization of the cell wall structure of the storage vacuoles allowing the release of free sugars.

The sugar profiles of hydrolyzed and control BM samples showed that for all hydrolyzed samples, the glucose content was higher than that of fructose whereas in the control BM samples their concentrations were equimolecular. This is indicative that the process produced a partial hydrolysis of some polysaccharides mainly composed of glucose. The result obtained with enzyme 7 in which sucrose and starch were totally degraded into glucose units must be highlighted.

3.2.2. Fermentation of the hydrolyzed bagasse musts

The above results are not enough to conclude about the benefit that the increase of sugars can exercise on ethanol yields. For this reason, the hydrolyzed musts were fermented. The ethanol obtained and its $Y_{p/s}$ values are shown in Table 2 for the seven enzymes at maximum hydrolysis times. In cases in which the

increase was slow (enzyme 4 and 7) or too fast (enzyme 6) musts with different hydrolysis time were fermented. The analysis of the results shows that the higher the concentration of sugars in the hydrolyzed must the higher the ethanol yield obtained, as expected. This fact rules out the possibility that among the hydrolysis products there are substances that could inhibit the biocatalyst or that are non-fermentable sugars, like it was mentioned by Sun and Cheng (2002).

(Table 2 here)

Summarizing, Figure 2 showed that it is interesting to note that the enzymatic hydrolysis of BM with enzymes Enzigrex or OptimaseCX255, during 2 h and 2.5 h, respectively, at their optimal conditions (Table1), allowed triplicating the ethanol obtained ($Y_{p/c}$ = 0.133 $g.g^{-1}$). Therefore, taking into account the ethanol yield with CM (see 3.1.), the total $Y_{p/c}$ reached by Strategy 1 in these conditions was 0.267 g g^{-1}. Comparing this value with the one corresponding to primary carrot processing, the increase of ethanol yield is 100 % and 51.3 L of bioethanol might be obtained from each ton of DC.

(Figure 2, here)

3.3 Strategy 2: Enzymatic hydrolysis previous to CM preparation

The scientific community has spent much effort in studying the application of enzymes in the vegetal juice industry in the past two decades, because it has proven to be a versatile tool for releasing components of tissue cells and intracellular compounds, thus improving juice productivity (Ovando and Waliszewski, 2005). In this way, enzymatic hydrolysis of DC previous to CM preparation was studied as a strategy to increase the extracted sugar and

consequently the ethanol yield. Enzymes 1, 2, 4 and 5 were used according to the supplier`s specifications and their availability.

Results showed that as time elapsed all enzymes increased the extracted volume of juice compared with the original CM. In spite of this, the juice volume extracted at different time of hydrolysis, its sugar concentration and the kinetics were different for each enzyme reaching an increase of about 50-60 % in some cases. Though the majority of the enzymes reached the maximum juice extraction at 2.5 h, enzyme 5 allowed obtaining such volume increase in only 1 h. In order to evaluate the efficiency of the different enzymes, the total sugar extracted after 2.5 h of hydrolysis expressed as (g kg^{-1}_{carrot}) is indicated in Table 3 together with the sugar concentrations of the must (g L^{-1}_{must}) used for fermentation. These results show that the higher yield in sugars extraction is obtained with the OptimaseCX255L biocatalyst during 1 h at enzyme optimal conditions. In this case, comparing with CM without enzyme treatment (see 3.1.), the extracted sugar was increased 3.5 times.

As in previous experiments, the analysis of carbohydrate composition of each hydrolyzed must showed that the sugars present were sucrose, fructose and glucose, all of them capable of being used by the yeast through alcoholic fermentation.

3.3.1. Fermentation of CM

The results of the fermentation of the CM obtained by different enzymatic hydrolysis are shown in Table 3 in comparison with those corresponding to original CM. In agreement with previous results (see 3.2.2), it can be observed that by

increasing the concentration of total sugars in the musts, the Yp/s values remain almost constant meaning that all substrates would be fermentable, but instead, $Y_{p/c}$ values vary substantially due to the different sugar extraction during must preparation. In fact, the $Y_{p/c}$ could be increased 3 times if DC was hydrolyzed with enzyme 5 before extracting the juice.

(Table 3 here)

In this way, Strategy 2 using enzyme OptimaseCX255 at its optimal conditions (see Table 1) during 1 h reached an $Y_{p/c} = 0.403$ g g^{-1}, as shown in Figure 2. So it allowed us to obtain 77.5 L of second-generation bioethanol from 1 t of DC turning itself into an interesting alternative which allows increasing the ethanol yield of discarded carrots. Thus, the remaining bagasse may be used for animal feed, as suggested by Castillo and Gallardo (1989).

It can be observed that though the ethanol yield of each ton of carrot may be minor to the one obtained with traditional crops, the high yield of biomass per each ha of carrot cultivation in this area gives an ethanol yield of 3,100 L ha^{-1}, similar to the yields of corn and sorghum, 2,960 L ha^{-1} and 3,010 L ha^{-1}, respectively (Sánchez and Cardona, 2008).

4. CONCLUSIONS

Enzymatic hydrolysis was adjusted to increase the ethanol yield and improve discarded carrot valorization. The two Strategies were efficient but enzymatic hydrolysis prior to CM preparation (Strategy 2) allowed duplicating the ethanol yield with respect to the must prepared without hydrolysis. Considering the

daily average of discarded amount, 4,650 L day^{-1} of second-generation bioethanol could be produced in this area. It is important to continue the global analysis of the process since after the distillation of the ethanol, a vinasse rich in water arises that might be used for animal feed as the fiber-rich bagasse remaining from the preparation of CM.

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FIGURE CAPTIONS

Figure 1: Reducing sugar (top graph) and total sugars (low graph) in BM during different enzymatic hydrolysis. (Ref.: 1: Rohament CL; 2: Rohalase OS; 3: Enzigrex; 4: IndiAge MAX L; 5: Optimase CX255L; 6: Spirizyme Fuel; 7: Fungamyl).

Figure 2: Schemes of the two different strategies proposed.

TABLE CAPTIONS

Table 1: Enzymes' specifications.

Table 2: Total sugars and ethanol obtained in hydrolyzed BM.

Table 3: Total sugars extracted from carrot and fermentation parameters of fermentation of CM previously hydrolyzed.

FIGURE 1

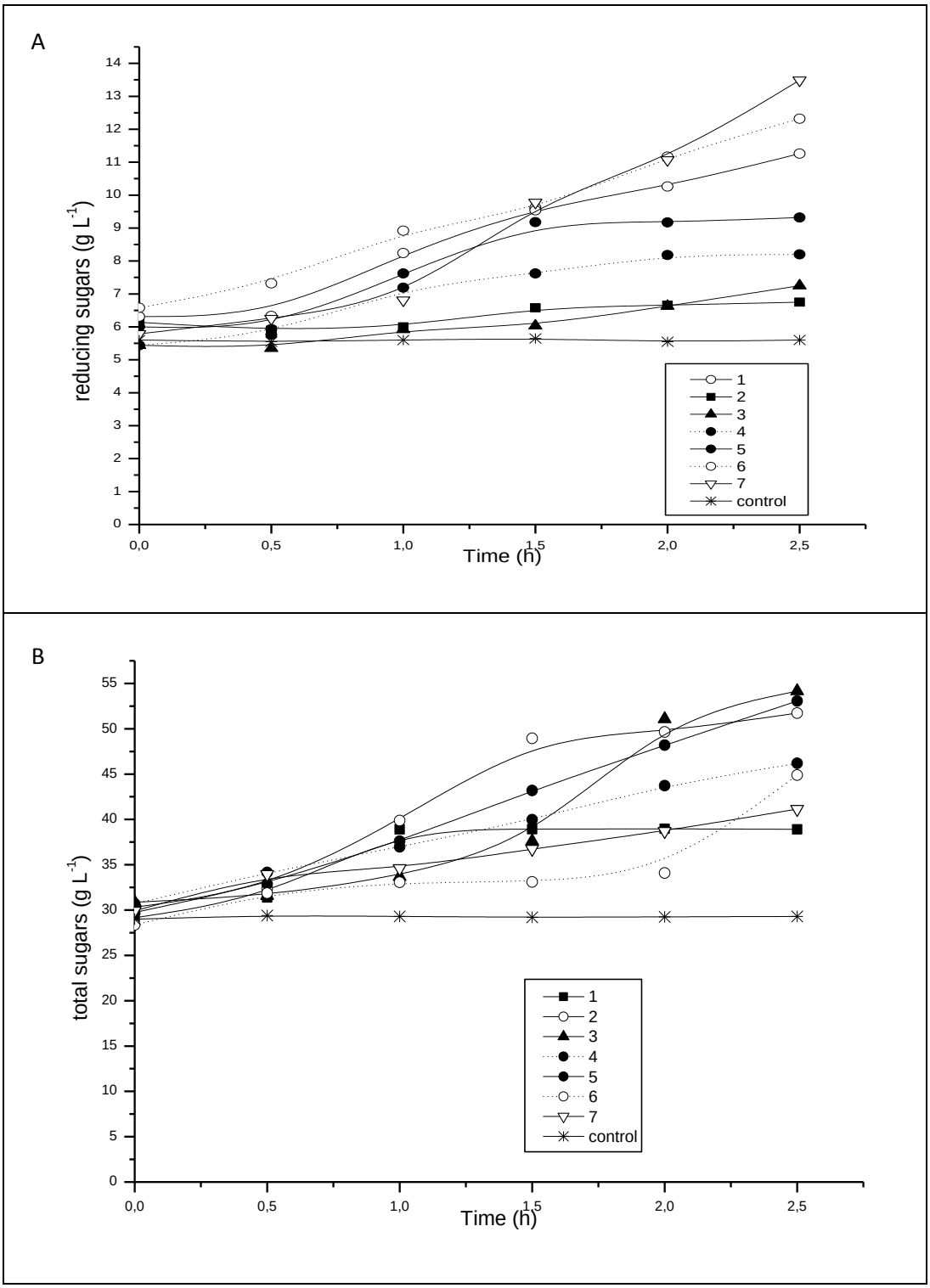
A
reducing sugars (g L-1)
Time (h)
1
2
3
4
5
6
7
control
B
total sugars (g L-1)
Time (h)
1
2
3
4
5
6
7
control

FIGURE 2

TWO STRAGIES TO INCREASE ETHANOL YIELD FORM DISCARDED CARROTS

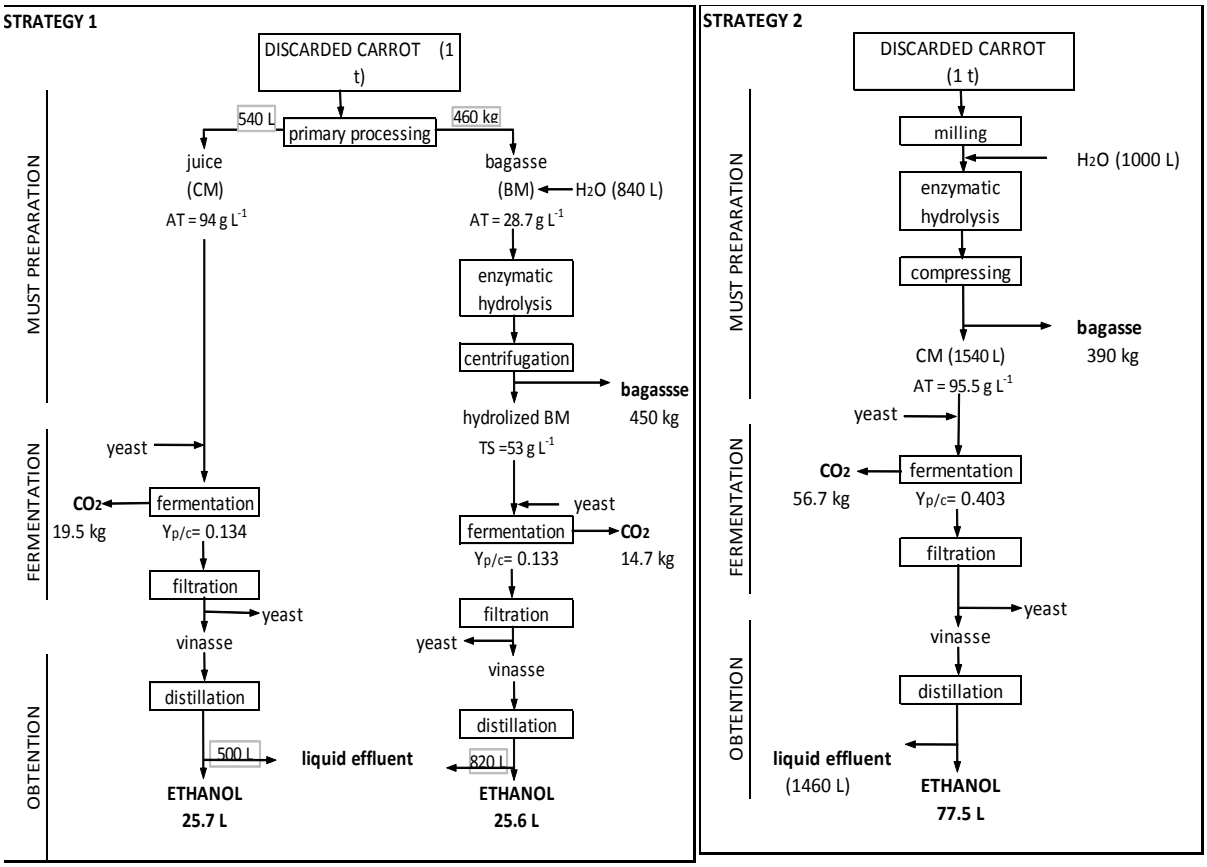

TABLE 1

Ref.	commercial name	enzymatic activity	Origin	Temp.	pH	Doses
1	Rohament CL	endo-1,4-β-glucanase	*Trichoderma reesei*	50 °C	5.0	0.05% v/v
2	Rohalase OS	cellulase, betaglucanase, xylanase	*Trichoderma reesei*	55 °C	5.5	0.05% v/v
3	Enzigrex	experimental cellulase	*Trichoderma reesei*	50 °C	5.5	0.02% w/v
4	IndiAge MAX L	Cellulase	*Trichoderma reesei*	50 °C	5.0	0.05% v/v
5	OptimaseCX255L	Thermostable Xylanase	*Trichoderma reesei*	70 °C	5.5	0.05% v/v
6	Spirizyme Fuel	Amyloglucosidase	*Aspergillus niger*	65 °C	4.5	0.25% v/v
7	Fungamyl	α-amylase	*Aspergillus oryzae*	55 °C	4.7	0.32% w/v

TABLE 2

ENZYME[a]	Incubation Time (h)	Total sugars ($g\ L^{-1}$)	Ethanol ($g\ L^{-1}$)	$Y_{p/s}$ ($g\ g^{-1}$)
-	-	28.7 ± 0.1	5.9 ± 0.2	0.205
1	1.5	48.9 ± 0.4	11.5 ± 0.5	0.235
2	1.5	38.6 ± 0.3	8.9 ± 0.4	0.230
3	2.0	51.1 ± 0.4	15.4 ± 0.5	0.301
4	2.0	43.7 ± 0.2	8.9 ± 0.4	0.204
4	2.5	46.2 ± 0.4	10.0 ± 0.3	0.216
5	2.5	53.1 ± 0.3	15.4 ± 0.2	0.290
6	2.0	34.1 ± 0.3	8.2 ± 0.2	0.241
6	2.5	44.9 ± 0.2	11.1 ± 0.3	0.247
7	2.0	34.7 ± 0.1	7.1 ± 0.2	0.205
7	2.5	41.1 ± 0.4	9.0 ± 0.2	0.219

[a]Table 2

TABLE 3

ENZYME[a]	TS ($g\ kg^{-1}_{carrot}$)	TS ($g\ L^{-1}_{must}$)	Ethanol ($g\ L^{-1}$)	$Y_{p/s}$ ($g\ g^{-1}$)	$Y_{p/c}$ ($g\ g^{-1}$)
-	42.3 ± 0.3	94.3 ± 0.7	37.1 ± 0.3	0.393	0.133
1	126.9 ± 0.7	84.6 ± 0.6	29.0 ± 0.5	0.343	0.290
2	106.5 ± 0.6	71.0 ± 0.3	27.3 ± 0.5	0.385	0.273
4	102.4 ± 0.6	68.2 ± 0.3	27.9 ± 0.5	0.409	0.279
5	150.2 ± 0.6	95.6 ± 0.6	40.3 ± 0.6	0.422	0.403

[a] Table 3