Scientific Bulletin, Series F, Biotechnologies, Vol. XVI, 2012
ISSN Online 2285-5521, ISSN-L 2285-1364

PRELIMINARY STUDY OF LACTIC ACID PRODUCTION FROM INULIN HYDROLYSATES USING *LACTOBACILLUS ACIDOPHILUS* LA-5

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Abstract

Lactic acid is widely used in the chemical industry, food industry, cosmetics, and pharmaceuticals. The main objective of the present work was chemical and enzymatic inulin hydrolysis from inulin rich feedstock (dahlia and Jerusalem artichoke) followed by hydrolysates fermentation to lactic acid using Lactobacillus acidophilus LA-5. Chemical hydrolysis of Jerusalem artichoke and dahlia flour was performed with concentrated H_2SO_4 for one hour at 100°C at different pH of the medium, and the amount of reducing sugars obtained varied between 0.10 to 0.51 g fructose/g dry weight flour for Jerusalem artichoke and between 0.099 to 0.448 g fructose/g dry weight flour for dahlia. The enzymatic hydrolysis was performed by varying the substrate concentration and the pH, and the amount of reducing sugars produced was between 0.439 to 0.56 g fructose/g dry weight flour for Jerusalem artichoke and between 0.435 to 0.522 g fructose/g dry weight flour for dahlia flour. The hydrolyzed substrates were then fermented with a commercial culture of Lactobacillus acidophilus LA-5 for 96 hours. Lactic acid production was monitored and recorded every 24 hours, measuring the pH and total acidity of the samples. The obtained results were then analyzed using Principal Component Analysis. An optimum number of 4 principal components are enough to explain 87.1% of data variation for calibration and 69.8% for validation. PC-1 is given by the acidity evolution, correlated with pH drop during fermentation. The lactic acid accumulation was maximum in the third day of fermentation (after 48 hours) for both dahlia and Jerusalem artichoke.

Key words: *acid hydrolysis, enzymatic hydrolysis, fructose, lactic acid fermentation, reducing sugars.*

INTRODUCTION

Inulin is a natural storage polymer found in the tubers of Jerusalem artichoke, dahlia, in roots of chicory, burdock, murnong, yacon, salsify, in bulbs of onion, leek, garlic, camas, in cereals such as rye, barley, etc [19].

Inulin is a polydisperse fructan that ranges in its degree of polymerization from 2 to 60, or higher [2].

The fructosyl units in inulin are linked by β (2-1) linkages with the polymer chains terminating in glucose residues. The tubers of Jerusalem artichoke, dahlia and chicory contain important amounts of inulin, those being most widely used natural sources of inulin. Van Loo determined the inulin content from Jerusalem artichoke tuber between 14-19% of fresh weight [19].

A variety of fractionated inulin can be produced from native inulin by physical, chemical or enzymatic processes, leading to products with a defined range of degree of polymerization and specific properties [13].

The acid hydrolysis of inulin has been investigated using sulphuric or hydrochloric acid [1, 12, 3].

Glibowski and Bukowska concluded that inulin chemical stability decreases in an acidic environment at pH under 4 due to the heating time and temperature increase and in a neutral and alkaline environment inulin is chemically stable independently of pH, heating time and temperature [6].

Inulin is hydrolysed by enzymes known as inulinases. Inulinases are classified into endo- and exoinulinases, depending on their mode of action. Endoinulinases (2,1-β-D-fructan fructanohydrolase; EC 3.2.1.7) hydrolyze

inulin by breaking the bonds between fructose units that are located away from the ends of the polymer network, to produce oligosaccharides. Exo-inulinases (β-D-fructohydrolase; EC 3.2.1.80), split terminal fructose units in sucrose, raffinose and inulin to liberate fructose [18, 11, 15].

The inulinases are produced by moulds, yeasts and bacteria. *A. niger* and *Kluyveromyces marxianus* are the most widely used for inulinase production. Among fungi, some well-known sources of these enzymes include *A. niger*, *Aspergillus ficuum*, *Chrysosporium pannorum* and *Penicillium purpurogenum*. Among yeasts, the best-known producers are *Kluyveromyces marxianus*, *Candida kefyr*, *Debaryomyces cantarellii* and *Pichia polymorpha*. These yeasts appear to produce only exo-inulinases whereas most inulin-hydrolyzing molds produce both endo- and exo-inulinases [16].

Szambelan and Nowak conducted the enzymatic hydrolysis with commercial inulinase (0.06 $mg \cdot g^{-1}$ and 0.12 $mg \cdot g^{-1}$ sugars) at pH 5.0 and with invertase (1.79 $mg \cdot g^{-1}$ and 17.90 $mg \cdot g^{-1}$ sugars) at the same pH. The results were best after 72 hours of enzymatic hydrolysis with inulinase dose of 0.12 $mg \cdot g^{-1}$ sugars and invertase dose of 17.90 $mg \cdot g^{-1}$ sugars [17].

Lactic acid (2-hydroxypropionic acid or 2-hydroxypropanoic acid), have applications in food, pharmaceutical, textile, leather, and other chemical industries. Lactic acid has gained many other industrial applications like biodegradable plastic production. Lactic acid is considered generally recognized as safe (GRAS) for use as food additives by the regulatory agencies like FDA in USA. In food industry, lactic acid is used as acidulant, flavoring or buffering agent or inhibitor of bacterial spoilage in a wide variety of processed foods, such as candy, breads and bakery products, soft drinks, soups, sherbets, dairy products, beer, jams and jellies, mayonnaise, and processed eggs, often in conjunction with other acidulants [8]. Besides high product specificity, as it produces a desired optically pure L-(+)- or D-(−)-lactic acid, the biotechnological production of lactic acid offers several advantages compared to chemical synthesis like low cost of substrates, low production temperature, and low energy consumption [7].

Until now, only a few studies have been made on lactic acid production from inulin rich feedstock, especially from Jerusalem artichoke tubers.

This study is focused on lactic acid production by a commercial culture of *Lactobacillus acidophilus* from acid and enzymatic inulin hydrolysates.

MATERIALS AND METHODS

Materials

The inulin rich feedstock used in this study was dahlia and Jerusalem artichoke flour. Dahlia flour was produced in the laboratory by cleaning and cutting the roots followed by freeze-drying using Alpha 1-4 LD Plus lyophilizator (Martin Christ, Germany) and finally grinding with VC2011 grinder (Victronic, PRC).

Jerusalem artichoke (*Helianthus tuberosus* L.) flour was kindly delivered to us by the Romanian company S.C. Hofigal Export-Import S.A.

Hydrolysis and fermentation

For chemical and enzymatic hydrolysis 2% and 6% (w/v) dahlia and Jerusalem artichoke flour were prepared by mixing with distilled water. The pH was measured using a pH meter S20 (Mettler Toledo, USA). The pH of the dahlia and Jerusalem artichoke flours were subsequently adjusted to 2, 4, and 6 using various concentrations of sulphuric acid.

For chemical hydrolysis, the solutions were adjusted to a pH value of 2 and 4 and then heated at 100 ± 2°C for one hour. For reducing sugars analysis, and inoculation with lactobacilli, the samples were cooled at 25°C and neutralized at pH = 6.0...6.1 using NaOH solution.

The enzymatic hydrolysis was conducted with commercial inulinase from *Aspergillus niger* (Novozyme A/S, Denmark). For enzymatic hydrolysis, the solutions were adjusted to a pH of 4 and 6, and then 0.7 ml/g substrate of commercial inulinase was added. The enzyme parameters were studied and found to be optimum at 60°C and 90 hours. After that, the

enzyme was inactivated by boiling the solutions at 100 ± 2°C for 10 minutes. The pH was adjusted at 6.0...6.1 after cooling at 25°C, using NaOH solution. All the hydrolysates were then pasteurized at 80°C for 30 minutes using Stericell 111 oven (MMM, Germany) and then cooled at 25°C.

The produced hydrolysates were coded according to Table 1.

Table 1. Hydrolysates codes used in the study

Code of the sample	Flour type	Hydrolysis type	Flour concentration (%)	Hydrolysate pH
Tha2-2	Jerusalem artichoke	acid	2	2
Tha2-4	Jerusalem artichoke	acid	2	4
Tha6-2	Jerusalem artichoke	acid	6	2
Tha6-4	Jerusalem artichoke	acid	6	4
The2-4	Jerusalem artichoke	enzymatic	2	4
The2-6	Jerusalem artichoke	enzymatic	2	6
The6-4	Jerusalem artichoke	enzymatic	6	4
The6-6	Jerusalem artichoke	enzymatic	6	6
Dha 2-2	dahlia	acid	2	2
Dha 2-4	dahlia	acid	2	4
Dha 6-2	dahlia	acid	6	2
Dha 6-4	dahlia	acid	6	4
Dhe 2-4	dahlia	enzymatic	2	4
Dhe 2-6	dahlia	enzymatic	2	6
Dhe 6-4	dahlia	enzymatic	6	4
Dhe 6-6	dahlia	enzymatic	6	6

The hydrolysates were then inoculated with 1% commercial culture of *Lactobacillus acidophilus* LA-5 (kindly provided by Christian Hansen, Denmark) and immediately incubated at 37°C using BF 4000 incubator (Binder, Germany). The fermentations were conducted in 100 ml flat bottom flasks. Lactic fermented samples were taken every 24 hours and analyzed for pH, reducing sugars and acidity.

Physico-chemical analysis

As analytical methods, Romanian standard determinations have been used for titrable acidity (g lactic acid/100 g product) and pH. The DNS (3, 5-dinitrosalicylic acid) method [10] was used for the quantitative analysis of the reducing sugar in inulin solutions. Adequately diluted samples were reacted with DNS acid. The intensity of developed color was measured using a 6505 UV-VIS spectrophotometer (Jenway, UK). Fructose was used for the establishment of a standard curve.

All the chemicals were of analytical grade.

Statistical analysis

Principal component analysis (PCA) is a variable reduction procedure. It is useful on the obtained data on a number of variables (possibly a large number of variables), when there is some redundancy in those variables. With minimal additional effort PCA provides a roadmap for how to reduce a complex data set to a lower dimension to reveal the sometimes hidden, simplified structure that often underlie it [14].

The experimental results were analyzed using Principal Component Analysis (PCA) with full cross-validation. Visualization of the results of PCA is usually achieved by plotting pairs of the first few PCs.

Principal Component Analysis was assessed using the Unscrambler X 10.1 software version from CAMO Software AS (Oslo, Norway). A principal component analysis (PCA) with full cross-validation was carried out in order to evaluate the influence of the studied parameters (type of carbon source, pH, and type of hydrolysis) on lactic acid production.

RESULTS AND DISCUSSION

After the production of chemical and enzymatic hydrolysates from Jerusalem artichoke and dahlia flour, the reducing sugars amounts were determined and the results are as follows.

The amount of reducing sugars obtained by enzymatic hydrolysis of Jerusalem artichoke flour were 0.44…0.56 g fructose/g d.w. flour.

Szambelan and Nowak [17] produced 166.32 g/kg reducing sugars when hydrolyzed with commercial inulinase the Jerusalem artichoke mashed tubers. Also, they produced 95.77 g/kg reducing sugars when treated the same mash tubers with commercial invertase.

The acid hydrolysis produced a high amount of reducing sugars only at pH of 2, the values being between 0.48...0.51 g fructose/g d.w. flour. At pH = 4 the acid hydrolysis of Jerusalem artichoke flour is not recommended, because the values obtained for reducing sugars are very low, between 0.10...0.11 g fructose/g d.w. flour.

Szambelan and Nowak [17], by doing the acid hydrolysis in the same conditions (pH=2, 100°C) produced 196.93 g/kg reducing sugars, and Glibowsky and Bukowska [6] an amount of 80g/100g, their determinations being performed on pure inulin.

The enzymatic hydrolysis of dahlia flour produced an amount of 0.43...0.52 g fructose/g d.w. flour, while the acid hydrolysis at pH = 2 liberated an amount of 0.40...0.45 g fructose/g d.w. flour.

At pH = 4 the amount of reducing sugars from dahlia hydrolysates is low, between 0.1...0.15 g fructose/g d.w. flour, comparable with those obtained for Jerusalem artichoke flour. The stability of inulin at high temperature and pH higher than 3, in the case of acidic hydrolysates, is the main cause for the production of the low amounts of reducing sugars [6].

Lactobacillus acidophilus is a homolactic bacterium, and is proved to be able to use the fructose as carbon source for lactic acid production [9].

The pH variations and the total acidity drop were measured every 24 hours. All the obtained data were analyzed using PCA analysis.

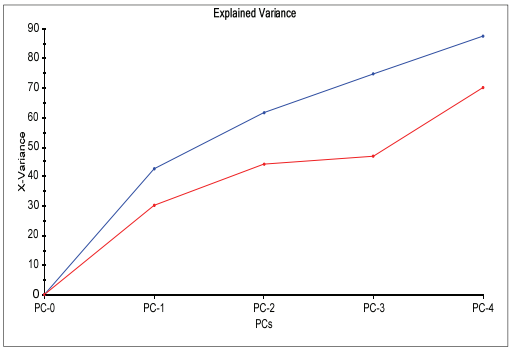

Fig. 1. The variance for the calibration and validation data.

As it can be seen from figure 1, 4 PCs are enough to explain over 87% of data variation and almost 70% for data validation.

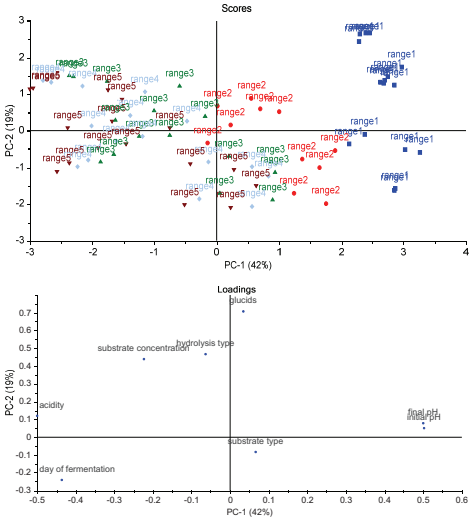

Fig. 2. Scores and loadings plots for PC-1 and PC-2 (range 1 – after 0 hours of fermentation, range 2 – after 24 hours of fermentation, range 3 – after 48 hours of fermentation, range 4 – after 72 hours of fermentation and range 5 – after 96 hours of fermentation).

In Fig. 2 the loadings plot and the scores plot are presented. The first PC (which explains 42% of data variation) is given by the acidity, pH and the day of fermentation of the samples. In this figure the samples were grouped after the day of fermentation. The total acidity of the samples is highly correlated with day of fermentation. After 24 hour of lactic acid fermentation, all the fermented substrates had a drop in pH values, around 5.00, especially for dahlia hydrolysates that had a decrease of pH around 4.00…4.50. Jerusalem artichoke acid and enzymatic hydrolysates with 2% concentration (The2-4, Tha2-4 and Tha2-2) had a slow decrease in pH, even after 48 hour of fermentation, due probably to the low concentration of substrate.

After 48 hours of fermentation, the studied inulin hydrolysates had a decrease of pH values until 4.50…3.70. Also the acidity slowly decreased after 48 hours of fermentation.

The second PC, which describes 19% of data variation, is given by fructose content of the samples.

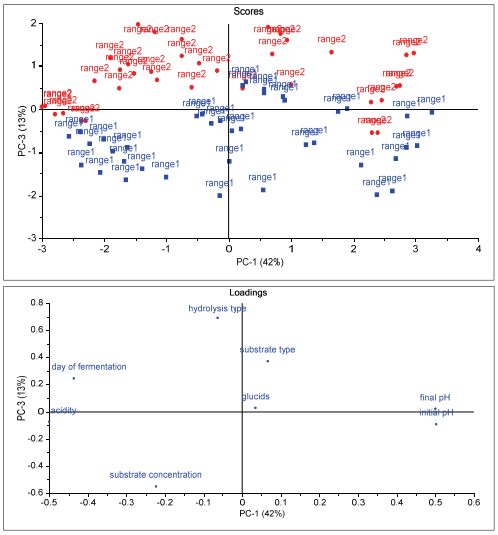

Fig. 3. Scores and loadings plots for PC-1 and PC-3 (range 1 – acidic hydrolysis, range 2 – enzymatic hydrolysis).

PC-3 explains 13% of data variation and is given by hydrolysis type: acidic or enzymatic. As it can be seen from figure 3, the hydrolysates obtained by enzymatic hydrolysis have higher concentrations in fructose (reducing sugars) and higher acidity after fermentation. The drop in pH of the samples during fermentation was faster.

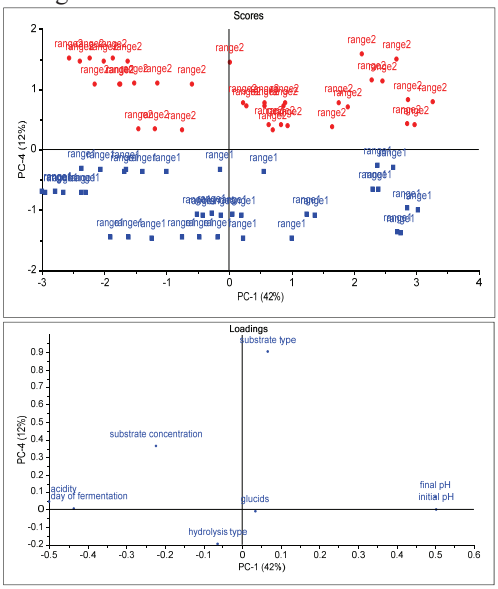

Fig. 4. Scores and loadings plots for PC-1 and PC-4 (range 1 – dahlia flour, range 2 – Jerusalem artichoke flour).

From figure 4 it can be observed that the PC-4 (which explains 12% of the data variation) is given by the type of the carbon source used in experiments. The dahlia tubers produced higher concentrations in lactic acid than Jerusalem artichoke. After 48 hours of fermentation, the 6% dahlia acid hydrolysates at pH=4 produced 6.3 g/l lactic acid, 2% dahlia acid hydrolysates at pH=4 produced 5.6 g/l, and 6% Jerusalem artichoke acid hydrolysates at pH=4 produced 5.4 g/l.

Regarding the conversion yield of reducing sugars to lactic acid, the values are presented in table 2.

Table 2. Jerusalem artichoke and dahlia conversion yield of reducing sugars to lactic acid.

Hydrolysate code	$Y_{P/S}$ at 24 h	$Y_{P/S}$ at 48 h
Tha2-2	0.505	0.609
Tha2-4	0.781	0.893
Tha6-2	0.508	0.631
Tha6-4	0.787	0.961
The2-4	0.438	0.627
The2-6	0.537	0.714
The6-4	0.542	0.717
The6-6	0.517	0.712
Dha 2-2	0.519	0.687
Dha 2-4	0.856	0.985
Dha 6-2	0.514	0.676
Dha 6-4	0.829	0.974
Dhe 2-4	0.530	0.741
Dhe 2-6	0.497	0.809
Dhe 6-4	0.544	0.821
Dhe 6-6	0.541	0.761

In the case of Jerusalem artichoke hydrolysates, the best conversion yield was obtained after 48 hours of fermentation for the 6% Jerusalem artichoke acid hydrolysates of pH = 4 (Tha6-4), followed by 2% Jerusalem artichoke acid hydrolysates of pH = 4 (Tha2-4), which had a very low initial amount of reducing sugars.

The hydrolysates with large amounts of reducing sugars had the lowest conversion yield after 48 hour of fermentation. This might be due to catabolic repression of the reducing sugars on the fermentative bacteria used in the study. For dahlia, after 24 hours of lactic acid fermentation, the conversion yield is best for 2% and 6% dahlia acid hydrolysates at pH=4 (Dha2-4 and Dha6-4) because of the low initial content in reducing sugars. After 48 hours of fermentation the conversion yield for Dha2-4 and Dha6-4 were the highest. This is due to the fact that the initial load of reducing sugars was lower. In the case of 2% and 6%

dahlia acid hydrolysates at pH=2 (Dha2-2 and Dha6-2), the conversion yields after 48 hours of fermentation were lower due to the fact that a good quantity of reducing sugars were not consumed by lactic bacteria. The obtained results are comparable with those obtained by Ge [5], who used *Aspergillus niger* SL-09 for inulinase production and *Lactobacillus* G-02 for lactic acid production. After 36 hours of fed-batch fermentation, the inulin bioconversion produced 120.5 g/l of lactic acid.

CONCLUSIONS

Lactic acid production from natural inulin rich substrates (dahlia and Jerusalem artichoke) was studied, using different methods of hydrolysis. The highest values of reducing sugars were obtained for the enzymatic hydrolysates and chemical hydrolysates at pH 2 of Jerusalem artichoke and dahlia flour. In the case of pH=4, the acid hydrolysates had the smallest amount of reducing sugars.

The pH=4 acidic hydrolysates after fermentation had the highest conversion yield. Dahlia yields for fermented acid hydrolysates at pH=4 (0.985 and 0.974) were better than the yields for Jerusalem artichoke (0.961 and 0.893) and better than all the other yields of fermented hydrolysates.

The lactic acid accumulation was maximum in the third day of fermentation (after 48 hours) for both dahlia and Jerusalem artichoke substrates.

Dahlia and Jerusalem artichoke roots can be successfully used as natural substrates for lactic acid bioproduction.

ACKNOWLEDGEMENTS

This work has benefited from financial support through the 2010 POSDRU/89/1.5/S/52432 project, „Organizing the national interest postdoctoral school of „Applied biotechnologies" with impact on Romanian bioeconomy", project co-financed by the European Social Fund through the Sectoral Operational Programme Human Resources Development 2007-2013.

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