DOI: https://doi.org/10.1016/j.renene.2013.06.006

FERMENTABLE SUGARS RECOVERY FROM GRAPE STALKS FOR BIOETHANOL PRODUCTION

I. Egüés [a], L. Serrano [a,*], D. Amendola [b], D.M. De Faveri [b], G. Spigno [b], J. Labidi [a]

[a] Chemical and Environmental Engineering Department, University of the Basque Country, Plaza. Europa 1, 20018, Donostia-San Sebastián, Spain

[b] Institute of Oenology and Agro-Food Engineering, Università Cattolica del Sacro Cuore, Via Emilia Parmense, 84-29122 Piacenza, Italy

* Corresponding author. Tel.: +34-943017125; fax: +34-943017140.
E-mail adress: luis.serrano@ehu.es (L. Serrano)

Abstract

Three different processes were investigated for the recovery of fermentable sugars from grape stalks: autohydrolysis at 121 °C before and after a pre-washing step and acid hydrolysis (2% H_2SO_4 w/w) after a pre-washing step. Moreover, optimal conditions of a charcoal-based purification process were determined by experimental design. All hydrolysates, with their corresponding synthetic liquors were used as fermentation substrates for the production of metabolites by the yeast: *Debaryomyces nepalensis* NCYC 1026. The main fermentation product was ethanol, where a maximum production of 20.84 g/L, a conversion yield of 0.35 g ethanol/g monomeric sugars and a productivity of 0.453 g/Lh were obtained from non-purified autohydrolysate liquor. In all cases, ethanol production and cell growth were better in non-purified liquors than in synthetic liquors. These results could be influenced by the presence of other sugars in the hydrolysates, with higher concentration in non-purified ones.

Keywords: Grape stalks, hydrolysis, sugars, purification, fermentation, ethanol

1. Introduction

Grape is one of the most important fruit crops and viticulture is one of the most important agricultural activities. The principal solid residues produced in grape juice and wine making processes are stalks and grape marc. Grape stalks are the skeleton of the grape bunch and consist in lignified tissues [1]. Its composition is tannins, lignin, cellulose and hemicelluloses principally. The upgrading of this by-product through the use of its components could entail economical and environmental improvements for the industry.

In concrete, hemicelluloses, which link the lignin and cellulose, are not being isolated for industrial use, but they have a high potential and they could be used in many applications. The hemicelluloses are made up of pentoses (β-D-xylose, α-L-arabinose), hexoses (β-D-mannose, β-D-glucose, α-D-galactose) and/or uronic acids (α-D-glucoronic, α-D-4-O-methylgalacturonic and α-D-galacturonic acids) where the hydroxyl groups of sugars can be partially substituted with acetyl groups [2]. Many studies have been focused in the hemicelluloses hydrolysates use to obtain biofuels, especially bioethanol, [3-5] and chemicals like xylitol [6], furfural [7] or lactic acid [8]. The composition and the concentration of hydrolysates rich in hemicelluloses, depend on the used raw material and the applied extraction process. Environmental-friendly technologies such as autohydrolysis process, which uses water as the only reagent, have gained interest for hemicelluloses sugars recovery. For that reason, in this study, hydrolysis process was studied using water as principal reagent for grape stalks fermentable sugars extraction.

Preliminary experiments were performed to determine autohydrolysis optimum conditions in order to recover the maximum fermentable sugars from grape stalks. Furthermore, previous studies [9] showed the presence of non-structural carbohydrates,

which are sugars derived from impregnate grape juice. For this reason, a raw material washing step before autohydrolysis process was also studied in order to know the contribution of these sugars.

Before fermentation study, toxic compounds which are typically generated during the hydrolysis process should be removed to improve the bioconversion of hydrolysates. Adsorption on activated charcoal could be an effective and low cost technique [10] depending on the optimization in the treatment variables. Therefore, in this study, an experimental design was applied to optimise the purification process with activated charcoal, investigating the influence of temperature, time, stirring rate and pH on the removal rates of lignin degradation products (LDP), colour (C) and sugars.

Finally, the hydrolysates were tested as fermentation substrates. For industrial applications, it is very important that the microorganism has the capacity to metabolize most of the sugars present in the hydrolysate, to obtain an efficient bioconversion of all the sugars into industrial interest metabolites. However, many wild-type yeast strains cannot utilize determinate sugars for their metabolism and are easily inhibited by toxic compounds generated during the hemicelluloses hydrolysis. For this reason, in this work the yeast *Debaryomyces nepalensis* NCYC 1026 was selected. In fact, this yeast, originally isolated from rotten apple, is known for its ability to metabolize both hexoses and pentoses sugars simultaneously and to grow also in non highly purified media [11].

2. Materials and Methods

2.1 Raw material and autohydrolysis conditions

The raw material used in this work was a mixture of grape stalks from two different Italian red cultivars, *Bonarda* and *Barbera*, kindly supplied by a wine-making factory in Piacenza (Italy) in the 2010 vintage. The samples were collected immediately

after the operation of pressing/destemming, oven dried at 60 ºC for 24 h, finally ground in a mill and sieved to obtain the 1– 4 cm size fraction.

Chemical composition of the raw material, given on an oven dry weight basis, was determined according to TAPPI standards [12] and bibliographic procedures. Ashes (T211 om-93), hot water (T264 cm-97) and 1% NaOH solubility (T212 om-98), ethanol–toluene extractives (T204 cm-97), lignin (T222 om-98), holocellulose [13], cellulose and hemicelluloses [14] contents were determined.

The hydrolysis treatments were carried out using unwashed grape stalks in a vertical autoclave, which supports a maximum temperature of 121 ºC and a maximum pressure of 198.67 kPa. The autohydrolysis process time was varied from 60 to 90, 105, 120 and 180 min, keeping constant the autohydrolysis temperature at 121 ºC and a solid/liquid ratio of 1:8 (w/v) in all experiments to find out the best conditions to get the maximum concentration of fermentable sugars.

For the raw material washing step, the grape stalks were suspended in distilled water (solid/liquid ratio of 1:10/ w/v) in an erlenmeyer flask and maintained under agitation at room temperature for 2 h. The solid phase was recovered by filtration, oven dried at 50 ºC and used then for hydrolysis process at determined optimum conditions.

Finally, the effect of sulphuric acid as autohydrolysis process catalyser (2% w/v) was studied to observe its effect on hemicellulosic stalks sugars extraction at autohydrolysis optimum conditions. All these experiments (schematised in Fig.1) allowed obtaining different sugar concentration liquors, providing information about the effect of different hydrolysis condition had on different fermentable sugars fractions recovery.

2.2 Hydrolysis liquor purification

A 2^4 full-factorial design with three coded levels leading 19 experiments was made to study the effect of four different activated charcoal treatment variables (with a

fixed charcoal:hydrolysate ratio 1:40): pH, stirring rate, contact time, and temperature. The range and levels (low -1, medium 0, high +1) of the variables investigated in this study were: pH 2-5-8, stirring rate 150-200-250 rpm; contact time 10-35-60 min; temperature 25-35-50 ºC. The conditions were chosen in according to previous studies and the work of Mussatto and Roberto [15]. Three assays in the centre point were carried out to estimate the random error of the experimental design. The removal rates of lignin degradation products (LDP) and colour (C) were taken as the responses of the experimental design.

All purification treatments were carried out in 250 ml Erlenmeyer flaks that contained 50 ml of unwashed grape stalk hydrolysate and were agitated on an orbital shaker during the purification treatment. After each treatment, the precipitate was removed by centrifugation at 5000 x g for 15 min and filtered with glass fibre filter before the HPLC analysis.

After hydrolysate purification, the pH of all liquors was adjusted at pH ~ 5.4 with NaOH 5 N and then sterilized in autoclave at 121 ºC for 15 min for their application in fermentable processes.

2.3 Fermentation conditions

D. nepalensis NCYC 1026 used in this study was supplied as freeze-dried strain by NCYC, National Collection of Yeast Cultures, UK. According to the given instructions, the yeast was activated in 10 ml malt extract (Oxoid), incubating for 5 days in an orbital shaker (HT Infors AG CH-4103, Switzerland) at 100 rpm and at 25 ºC. The activated yeast was then divided into 3 aliquots and 50 mL of fresh malt extract was added to each one. For three days, each day 20 ml of new fresh malt extract were added until a concentration of $1.07x10^8$ cell/ml was obtained. After that, the strain was centrifuged, washed and grown in two different synthetic media supplemented by similar sugars

composition of the hydrolysis liquors obtained in this study: 30 g/L of glucose + 30 g/L of fructose (with 6.5 g/L yeast nitrogen base, YNB from Difco™) for L_1, its purified L'_1 liquor, L_2 and its purified L'_2 liquor, and 12 g/L glucose + 2.50 g/L fructose (with 6.5 g/L YNB) for L_3 and its purified L'_3 liquor. After three days of growth a known volume of the first semi-synthetic culture medium was transferred into 50 ml of L_1, L'_1, L_2, and L'_2 experimental liquors, corresponding to $1x10^6$ cell/ml initial concentration, whereas for the second culture medium a known volume was transferred into 50 ml of L_3 and L'_3 liquors always to obtain a $1x10^6$ cell/ml initial concentration. Additional synthetic liquors (B_1, B_2 and B_3), with the same sugars concentration of L_1, L_2 and L_3 hydrolysates, were also prepared with standard sugars (Carlo Erba, Italy) to compare the fermentation yield and the growth in synthetic liquors with original liquors. All the inoculated liquors (in 250 ml Erlenmeyer flasks) were incubated in the orbital shaker at 140 rpm and 25 ºC during the experiments. Each trial was carried out in duplicate.

Aliquots of 1 mL were periodically collected from different incubated liquors to observe the cell growth, sugars consumption and metabolite productions. For this objective, the aliquots were centrifuged at 8200 x g for 10 second to precipitate the yeast and to obtain the supernatant.

2.4 Analytical procedures

To determine the lignin degradation products, the samples were diluted (1:1000) in distilled water and analyzed at 280 nm by a UV-1601 Shimadzu spectrophotometer. For the colour determination, the samples were diluted (1:50) and analyzed at 440 nm.

The cell growth was evaluated measuring the optical density of culture at 600 nm (OD_{600}), whereas the relation between absorbance and cell concentration (evaluated by cell counting in Burker camera under optic microscope) was previously determined to obtain a calibration curve.

The sugars consumption was evaluated from supernatant using Megazyme kit assays for D-Glucose, D-Fructose and D-Xylose determination, whereas the by-products and other sugars components were analyzed by High Performance Liquid Chromatography (HPLC) Jasco LC Net II/ADC equipped with a refractive index detector and a photodiode array detector. A Phenomenex Rezex ROA HPLC column (300 mm x 7.8 mm) was used for the experiment, and 0.005 N H_2SO_4 prepared with 100 % deionised and degassed water was used as mobile phase (0.35 mL/min flow, 40 ºC and injection volume 40 µL). High purity standards of arabinose, galacturonic acid, lactic acid, xylitol and ethanol (supplied by Fluka, Sigma Aldrich and Panreac), were used for the calibration curves.

3. Results and Discussion

The composition of the grape stalks (weight percentage) used in this work was: 22.61±2.14% extractives, 32.35±0.31% lignin, 12.19±0.52% cellulose, 26.43±0.42% hemicelluloses (13.35% glucose and 13.08% fructose) and 6.11± 0.43% ashes. This high fructose concentration in the raw material derived from grape juice which impregnated stalks during destemming operations.

3.1 Autohydrolysis

Unwashed grape stalks were used to find out the optimum conditions for maximum fermentable sugars extraction. Table 1 shows the results of sugars concentrations in the liquors of the trials aimed to study the best conditions of autohydrolysis in order to maximize the glucose and fructose content.

All the liquors presented an acid pH value due to the organic acid formed in the autohydrolysis process. The optimal conditions chosen to use the autohydrolysis liquor as a fermentative medium were 121 ºC, 90 min, 1/8 because this experiment presented the highest fermentable sugar concentration.

3.2 Autohydrolysis liquor purification experimental design

Autohydrolysis liquor contained, in addition to fructose and glucose as the major sugars, several compounds that are toxic to yeasts, namely, acetic acid, furfural, hydromethylfurfural (HMF), and lignin degradation products.

The results of the experimental design (Table 2) showed that the removal of colour and lignin degradation products was dependent on the conditions employed in the treatment of the unwashed grape stalk hydrolysate with activated charcoal.

The hydrolysate colour is directly related to the presence of lignin degradation products (phenolic compounds), so a loss of colour in the treatment with activated charcoal is obtained by removing these compounds from the hydrolysate.

Lignin degradation products and colour removal were strongly influenced by the temperature and pH. pH 2 produced better results than pH 5 and 8, probably as a result of the low formation of phenolate ions at low pH and the fact that these ions are poorly adsorbed on activated charcoal [16]. High temperatures promoted an increase in the density of the packing of phenolic molecules in the activated charcoal pores [17] producing a high removal of phenolic compounds, so the colour intensity of the hydrolysate also decreased.

The conditions presented in the experiment 11 (pH=2, 250 rpm, 10 min, 50 ºC) were selected as the best conditions for removal the colour and lignin degradation products with the objective of the application of hydrolysate in fermentation processes after the purification process.

The selected purification conditions were applied to the liquors from hydrolysis of grape stalks, hydrolysis of washed grape stalks and acid hydrolysis of washed grape stalks. The results are presented in Table 3.

As it can be observed, the purification process at optimal conditions applied into different grape stalks hydrolysates allowed to reduce the toxic components represented by the decrease in absorbance mainly at 280 nm.

3.3 Sugars consumption and by-products determination

The sugars consumption and by-products obtained from *D. nepalensis* growth in all liquors were represented in Figs. 2-4. Table 4 summarized the ethanol maximum production yield, volumetric productivities and biomass yield.

As it can be observed by Figs 2-4, the main product obtained from *D. nepalensis* growth in the studied liquors was ethanol, followed by lactic acid at less concentration and xylitol in the case of xylose presence (Fig. 4).

As it has been shown in Table 4, the highest ethanol yield ($Y_{E/S}$ and $Y_{E/St}$) as well as the productivity (Q_p), was obtained from L_1 hydrolysate (taking into account the consumption of glucose fructose and xylose for ethanol production) followed by L_2 and finally from L_3. Therefore, the highest sugars extraction and the highest ethanol production was obtained from non washed grape stalks autohydrolysis process (with maximum of 20.84±1.25 g/L ethanol production at 46 h) being the best way for grape stalks fermentable sugars bioconversion using *D. nepalensis*.

As it can be observed in Figs. 2-4, between purified and non-purified liquors, the ethanol production was slightly higher in purified liquor only at initial times, whereas at higher fermentable times the non-purified liquors achieved best results. This suggested that the toxic component existing in these liquors did not inhibit greatly the *D. nepalensis* fermentation and therefore the metabolite production.

On the other hand, the ethanol production, yield and productivity were higher in hydrolysates than in synthetic liquor, suggesting that the hydrolysates had better conditions which enhanced ethanol production, even without being purified. Besides

glucose and fructose, the presence of galacturonic acid (highlighted in L_2 and L'_2 liquors, Figures 3b and c) and arabinose were detected by HPLC in all grape stalks hydrolysates. The galacturonic acid presence could indicate the presence of pectins in the hydrolysates, which can be used by the yeast as source of carbon [18]. Moreover, it was observed that arabinose decreased during the fermentation process. The presence of these minor components in hydrolysates could have improved the ethanol production in hydrolysates comparing with synthetic liquor.

As could be observed in Fig. 4, the production of ethanol was produced initially by the consumption of glucose and fructose. Furthermore, a second stage of maximum ethanol production was observed at higher fermentation time together with xylose consumption, achieving slightly higher ethanol production and also a maximum xylitol production. These results suggest that the xylose monomer was mainly used by the yeast for ethanol production with slight xylitol production. This observation is in agreement with the study of Sánchez et al. [19] who reported similar tendency of sequential consumption of glucose and xylose using mixtures of these sugars in culture medium.

In bibliography, the maximum ethanol concentration of 35.8±2.3 g/L with product yield of 0.27±0.03 g/g substrate and productivity of 0.36±0.04 g/L h using *D. nepalensis* with initial concentration of 200 g/L glucose was obtained by Kumar and Gummadi [18]. Ethanol production using 50 g/L sugars rice hull hydrolysate, constituted mainly by 35 g/L glucose followed by xylose and arabinose, was studied using *Sacharomycess* and *Spathaspora yeast* by Cunha-Pereira et al. [20]. In that study a maximum ethanol production of 15 g/L at volumetric productivities of 0.38 g/Lh was obtained. A batch culture using *P. tannophilus* with sugarcane bagasse hemicellulosic hydrolysate constituted mainly by 45 g/L of xylose, was also developed by Cheng et al. [21], achieving 19 g/L ethanol with 0.57 g/Lh productivity. The results obtained in this

study, mainly using non-purified grape stalks autohydrolysate L_1, could be considered under acceptable values.

3.4 Yeast growth

The cell growth of *D. nepalensis* in different liquors, as well as the monomeric sugars consumption (respect to the initial concentration) were shown in Figure 5. Table 4 summarizes different biomass yields.

In Fig.5a, it can be observed that the cell growth of *D. nepalensis* was higher in L_1 and L'_1 than in B_1 synthetic liquor justifying also the better metabolite production observed in such liquors in previous section. The biomass yield $Y_{B/S}$ (expressed as $x10^9$ cell biomass per g monomeric sugar consumed), is higher in purified liquors (L'_1 and L'_2) than non-purified and their corresponding synthetics liquors. This justified the better ethanol production observed in these liquors until maximum ethanol production observed in previous section. In Figure 5b and c, it can be observed the same tendency, lowest growth in synthetic liquor than hydrolysates.

Respect to the non-purified and purified liquor, between L_1-L'_1 and between L_2 and L'_2, the growth followed the same tendency whereas between L_3-L'_3 the growth was better in non-purified liquor.

4. Conclusions

Grape stalks hydrolysis processes allowed obtaining liquors with different concentration of fermentable sugars which were used by *D. nepalensis* for industrial metabolite productions. The main product obtained in the *D. nepalensis* growth was ethanol, followed by lactic acid and xylitol in the presence of xylose, produced mainly at glucose exhaustion. In all liquors, ethanol production and cell growth was better in grape stalks hydrolysates than synthetic liquor, and the ethanol production was higher in non-

purified liquor. These results could be influenced by the presence of other sugars in hydrolysates.

Acknowledgements

The authors would like to thank the Juan de la Cierva program (JCI-2011-09399) of Spanish Ministry of Science and Innovation, Department of Education, Universities and Investigation of the Basque Government (Grant of I.E., ref BFI09.164), Agrisystem Doctoral School of the Università Cattolica del Sacro Cuore, Italy-Spain Integrated Actions Program (project IT2009-0054) and Progetto Ager grant no 2010-2222 for supporting this work.

References

[1] Ping L, Brosse N, Sannigrahi P, Ragauskas A. Evaluation of grape stalks as a bioresource. Ind Crops Prod 2011;33:200-4.

[2] Gírio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques S, Bogel-Łukasik R. Hemicelluloses for fuel ethanol: a review. Bioresour Technol 2010;101:4775-4800.

[3] Prasad S, Singh A, Joshi HC. Ethanol as an alternative fuel from agricultural, industrial and urban residues. Resour Conserv Recy 2007;50:1–39.

[4] Kazi FK, Fortman JA, Anex RP, Hsu DD, Aden A, Dutta A, Kothandaraman G. Techno-economic comparison of process technologies for biochemical ethanol production from corn stover. Fuel 2010;89:S20–8.

[5] Kadam KL, McMillan JD. Availability of corn stover as a sustainable feedstock for bioethanol production. Bioresour Technol 2003;88,17–25.

[6] Silva CJSM, Mussatto SI, Roberto IC. Study of xylitol production by Candida guilliermondii on a bench bioreactor. J Food Eng 2006;75:115–9.

[7] Yemis O, Mazza G. Acid-catalyzed conversion of xylose, xylan and straw into furfural by microwave-assisted reaction. Bioresour Technol 2011;102:7371-8.

[8] Sánchez C, Egüés I, García A, Llano-Ponte R, Labidi J. Lactic acid production by alkaline hydrothermal treatment of corn cobs. Chem Eng J 2012;181–182:655– 660.

[9] Spigno G, Pizzorno T, De Faveri DM. Cellulose and hemicelluloses recovery from grape stalks. Bioresour Technol 2009;99:4329-4337.

[10] Mussatto SI. Influência do Tratamento do Hidrolisado Hemicelulósico de Palha de Arroz na Producao de Xilitol por *Candida guilliermondii*. MSc. Dissertation, Faculty of Chemical Engineering of Lorena, Brazil 2002.

[11] Kumar S, Gummadi SN. Purification and biochemical characterization of a moderately halotolerant NADPH dependent xylose reductase from Debaryomyces nepalensis NCYC 3413. Bioresour Technol 2011;102:9710-7.

[12] TAPPI Standards, 2007. TAPPI Test Methods, Atlanta.

[13] Wise LE, Murphy M, D'Adieco AA. Chlorite holocellulose, its fractionation and bearing on summative wood analysis and on studies on the hemicelluloses. Paper Trade J 1946;122 (2):35–43.

[14] Rowell, R. The chemistry of solid wood: based on short course and symposium sponsored by the division of cellulose, paper, and textile chemistry. In: The 185th Meeting of the American Chemical Society, Seattle, Washington, 1983;20–25:70–2.

[15] Mussatto SI, Roberto I. Optimal experimental condition for hemicellulosic hydrolyzate treatment with activated charcoal for xylitol production. Biotechnol Prog 2004;20:134-9.

[16] Talcott ST, Howard LR. Phenolic autoxidation is responsible for color degradation in processed carrot puree. J Agric Food Chem 1999;47:2109-2115.

[17] Ravi VP, Jasra RV, Bhat TSG. Adsorption of phenol, cresol isomers and benzyl alcohol from aqueous solution on activated carbon at 278, 298 and 323 K. J Chem Technol Biotechnol 1998;71:173-9.

[18] Kumar S, Gummadi SN. Metabolism of glucose and xylose as single and mixed feed in Debaryomyces nepalensis NCYC 3413: production of industrially important metabolites. Appl Microbiol Biotechnol 2011;89:1405–1415.

[19] Sánchez S, Bravo V, Castro E, Moya AJ, Camacho F. The fermentation of mixtures of D-glucose and D-xylose by Candida shehatae, Pichia stipitis or Pachysolen tannophilus to produce ethanol. J Chem Tech Biot 2002;77:641-8.

[20] Cunha-Pereira F, Hickert LR, Sehnem NT, Souza-Cruz PB, Ros CA, Ayub MAZ. Conversion of sugars present in rice hull hydrolysates into ethanol by Spathaspora arborariae, Saccharomyces cerevisiae, and their co-fermentations. Bioresour Technol 2011;102:4218–4225.

[21] Cheng KK, Cai BY, Zhang JA, Ling HZ, Zhou YJ, Ge JP, Xu JM. Sugarcane bagasse hemicellulose hydrolysate for ethanol production by acid recovery process. Biochem Eng J 2008;38:105–9.

List of tables

Table 1

Experiments	Glucose (g/L)	Fructose (g/L)	pH
60 min	5.50±0.50	6.80±0.60	3.92±0.10
90 min	8.30±0.80	9.60±0.50	3.98±0.10
105 min	7.40±1.10	8.70±1.00	3.89±0.10
120 min	7.05±1.25	8.40±1.15	3.86±0.10
180 min	8.20±1.50	8.90±1.10	3.87±0.10

Table 2

Experiment	[a]X_1	[b]X_2	[c]X_3	[d]X_4	CR (%)	LDPR (%)
1	-1	-1	-1	-1	65.5	91
2	+1	-1	-1	-1	43.7	87.4
3	-1	+1	-1	-1	51.5	89.9
4	+1	+1	-1	-1	53.7	92.4
5	-1	-1	+1	-1	44.8	87.3
6	+1	-1	+1	-1	50.8	87.9
7	-1	+1	+1	-1	54.9	87.4
8	+1	+1	+1	-1	44.1	80.5
9	-1	-1	-1	+1	75.1	74.2
10	+1	-1	-1	+1	60.3	80
11	-1	+1	-1	+1	83.4	100
12	+1	+1	-1	+1	43.6	73.2
13	-1	-1	+1	+1	80.6	100
14	+1	-1	+1	+1	71.3	95.8
15	-1	+1	+1	+1	74.9	100
16	+1	+1	+1	+1	66.1	95.7
17	0	0	0	0	59	80.4
18	0	0	0	0	53.2	81.1
19	0	0	0	0	45.3	80.6

[a]X_1: pH normalized; [b]X_2: stirring rate normalized; [c]X_3: contact time normalized; [d]X_4: temperature normalized; ranges: pH 2-5-8, stirring rate 150-200-250 rpm; contact time 10-35-60 min; temperature 25-35-50 ºC.

Table 3

Liquors	Absorbance		Concentration (g/L)		
	Colour	LDP	Glucose	Fructose	Xylose
L_1	0.87	0.53	30.96±1.19	30.70±1.50	0
L'_1	0.14	0.00	32.00±1.00	30.00±1.20	0
L_2	0.75	0.53	6.01±0.10	5.96±0.28	0
L'_2	0.07	0.09	6.05±0.13	6.15±0.20	0
L_3	0.38	0.69	12.22±0.33	2.57±0.20	9.78±1.19
L'_3	0.009	0.17	10.90±0.10	2.54±0.05	9.27±0.10

Table 4

Liquor	Sugars concentration	Fermentation Yields					
	(g/L)	Max ethanol production (g/L)	$Y_{E/S}$ (g/g)	$Y_{E/St}$ (g/g)	Q^p (g/Lh)	$Y_{B/S}$ (10^9cell/g)	$Y_{E/B}$ (g/10^9cell)
B_1	60.00	13.14±1.50	0.22	-	0.07	8.03	0.03
L_1	61.66	20.84±1.25	0.34	0.14	0.45	10.57	0.03
L'_1	62.00	13.16±1.10	0.30	0.09	0.45	14.39	0.02
B_2	12.00	1.90±0.25	0.18	-	0.09	16.17	0.01
L_2	11.97	2.66±0.30	0.22	0.01	0.12	25.56	0.01
L'_2	12.20	2.38±0.17	0.19	0.01	0.11	28.36	0.01
B_3	24.50	3.75±0.20	0.15 (0.26)[1]	-	0.07	8.86 (14.96)[a]	0.02
L_3	24.57	5.20±0.35	0.21 (0.35)[1]	0.02	0.07	19.21 (31.91)[a]	0.01
L'_3	22.71	1.86±0.35	0.08 (0.14)[1]	0.01	0.06	11.41 (19.27)[a]	0.01

$Y_{E/S}$: ethanol yield (g ethanol per g monomeric sugar consumed)
$Y_{E/St}$: ethanol yield (g ethanol per g of dry hydrolysed stalks)
Q^p: volumetric productivities (g/L of ethanol per hour)
$Y_{B/S}$: biomass yield (x10^9cell biomass per g monomeric sugar consumed)
$Y_{E/B}$: ethanol yield (g ethanol per x10^9cell biomass)
[a]: Yield taking into account the production of ethanol from glucose and fructose (without xylose)

List of figures:

Fig. 1

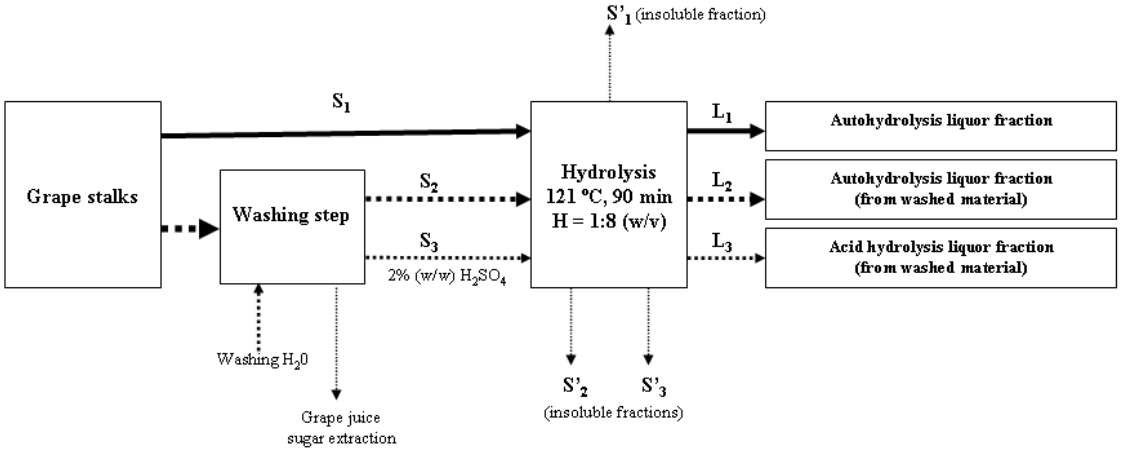
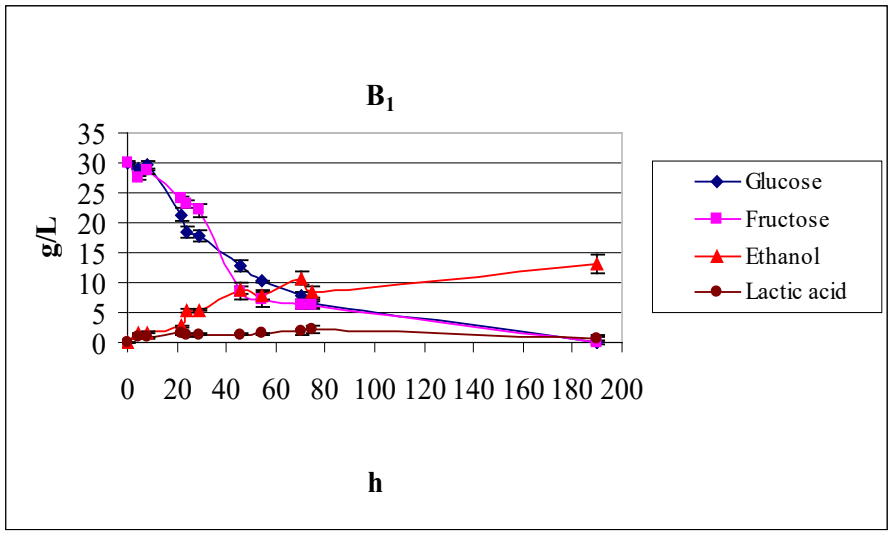
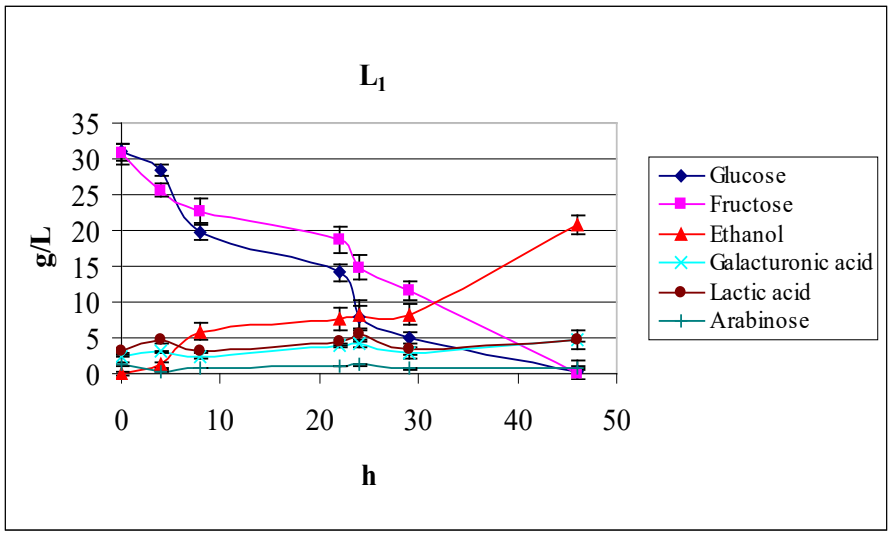

S'1 (insoluble fraction)
S1
Grape stalks
Washing step
S2
S3
2% (w/w) H2SO4
Hydrolysis
121 °C, 90 min
H = 1:8 (w/v)
L1
L2
L3
Autohydrolysis liquor fraction
Autohydrolysis liquor fraction
(from washed material)
Acid hydrolysis liquor fraction
(from washed material)
Washing H20
Grape juice
sugar extraction
S'2
S'3
(insoluble fractions)

Fig. 2

a)


B1
g/L
h
Glucose
Fructose
Ethanol
Lactic acid

b)


L1
g/L
h
Glucose
Fructose
Ethanol
Galacturonic acid
Lactic acid
Arabinose

c)

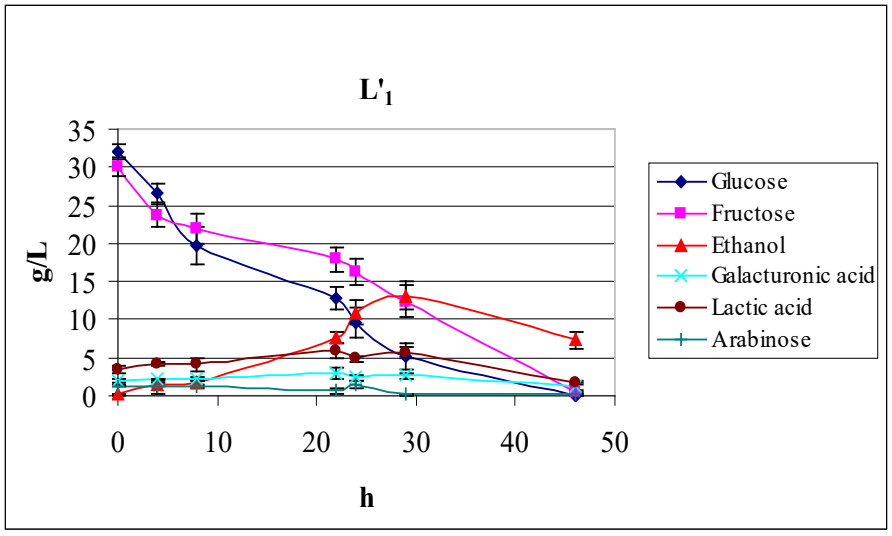
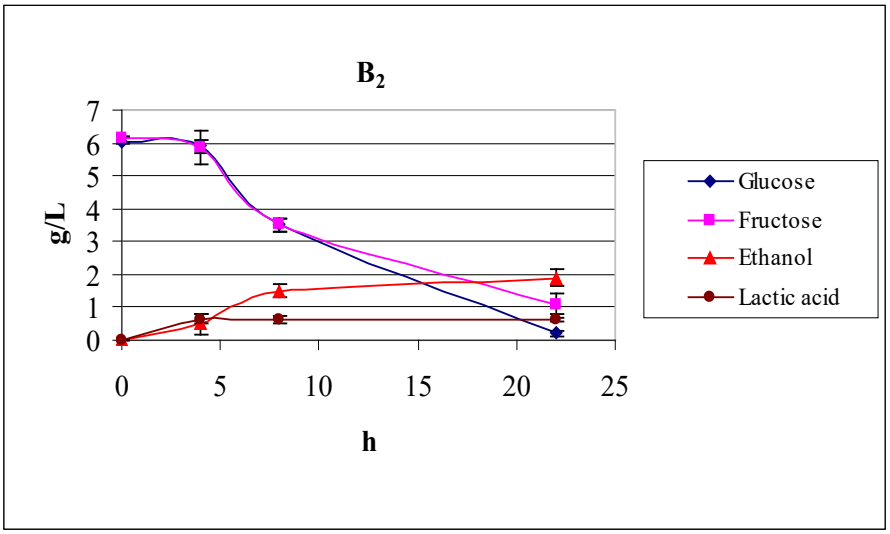
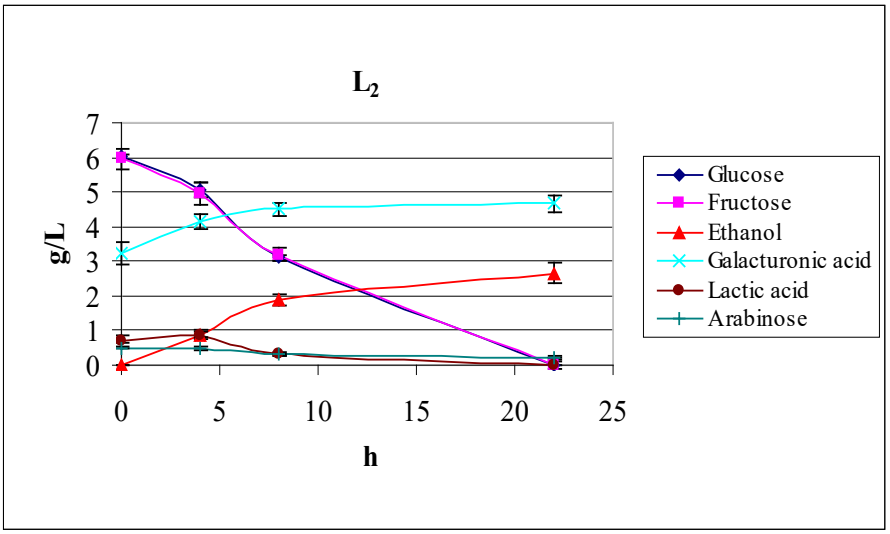

L'1
g/L
h
Glucose
Fructose
Ethanol
Galacturonic acid
Lactic acid
Arabinose

Fig. 3

a)


B2
g/L
h
Glucose
Fructose
Ethanol
Lactic acid

b)


L2
g/L
h
Glucose
Fructose
Ethanol
Galacturonic acid
Lactic acid
Arabinose

c)

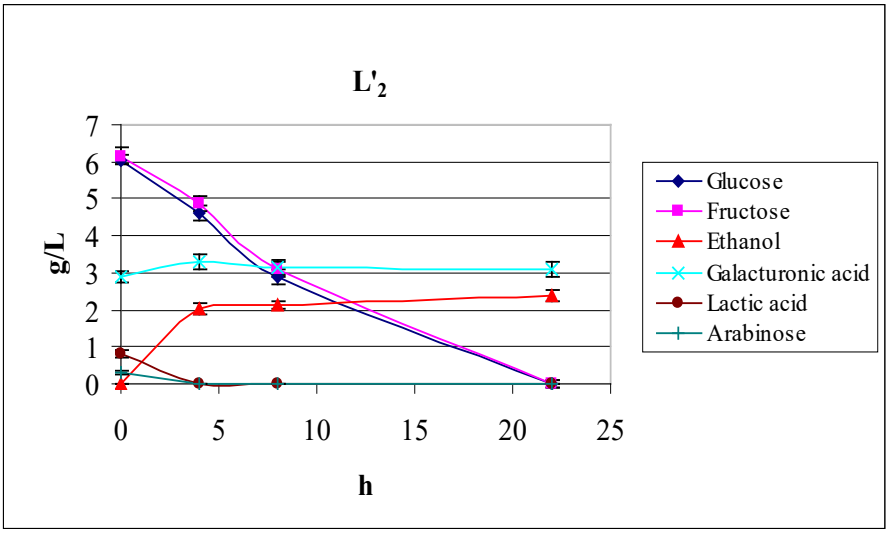
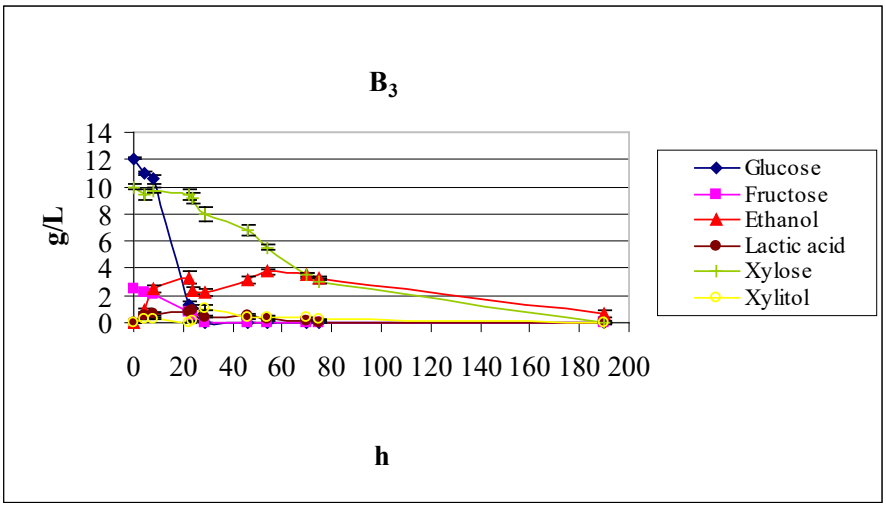
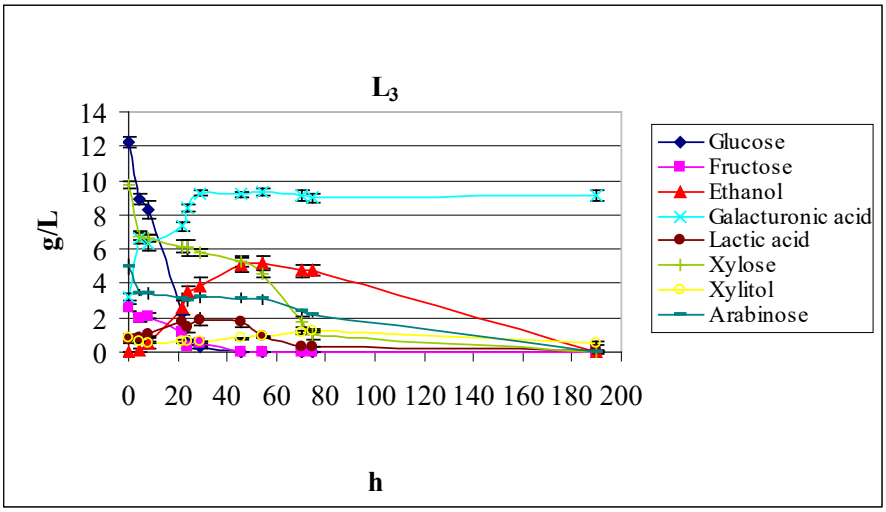

L'2
g/L
h
Glucose
Fructose
Ethanol
Galacturonic acid
Lactic acid
Arabinose

Fig. 4

a)


B3
g/L
h
Glucose
Fructose
Ethanol
Lactic acid
Xylose
Xylitol

b)


L3
g/L
h
Glucose
Fructose
Ethanol
Galacturonic acid
Lactic acid
Xylose
Xylitol
Arabinose

c)

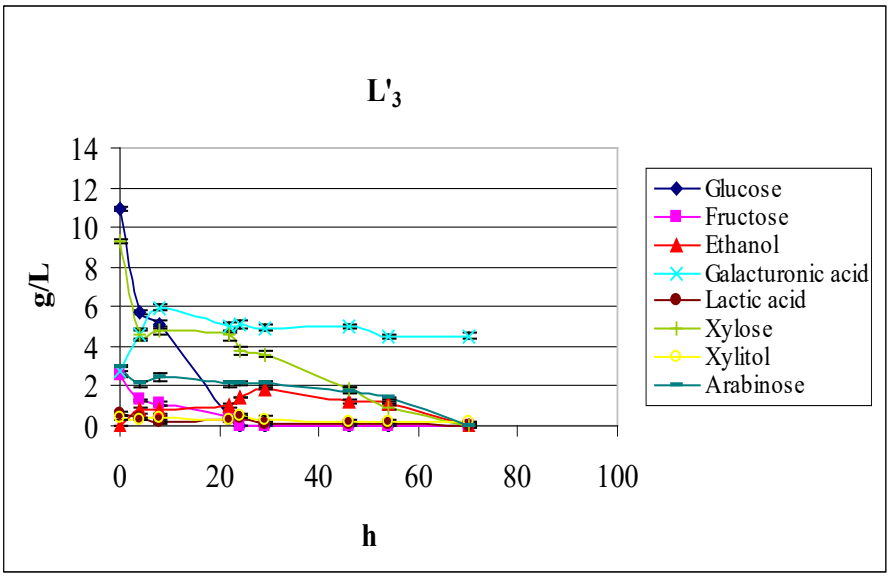
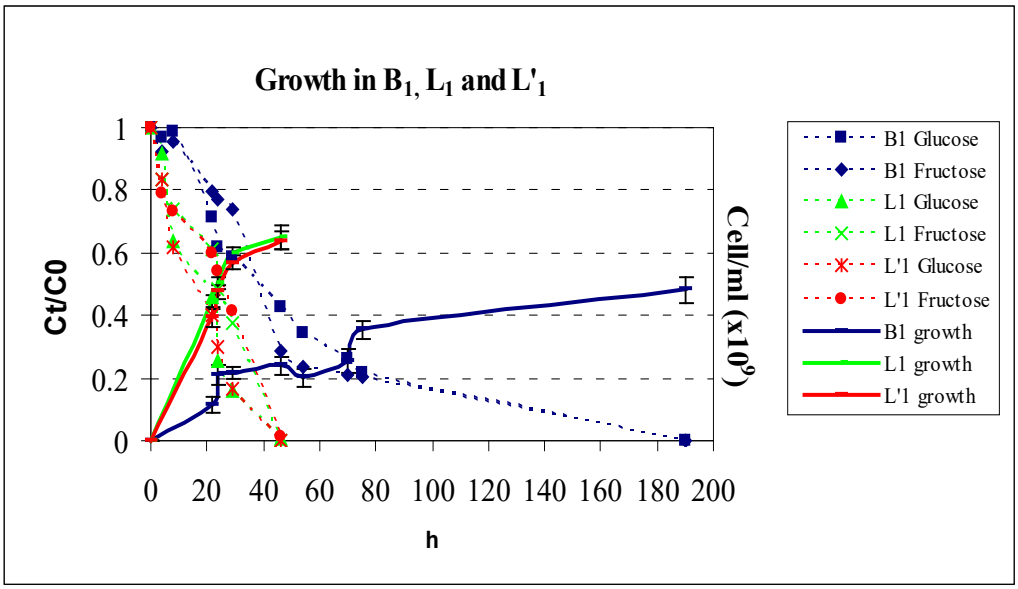
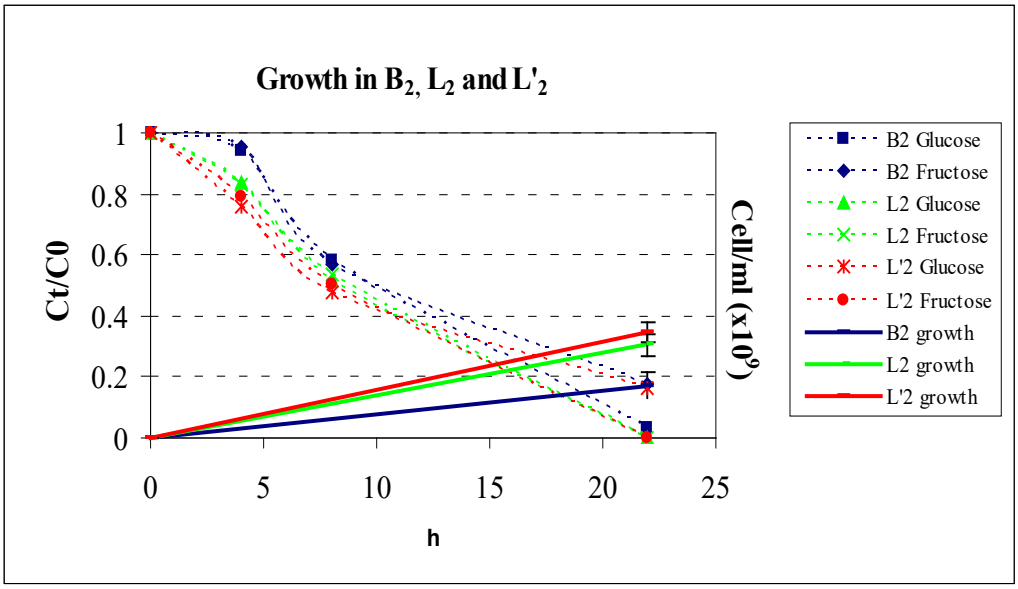

L'3
g/L
h
Glucose
Fructose
Ethanol
Galacturonic acid
Lactic acid
Xylose
Xylitol
Arabinose

Fig. 5

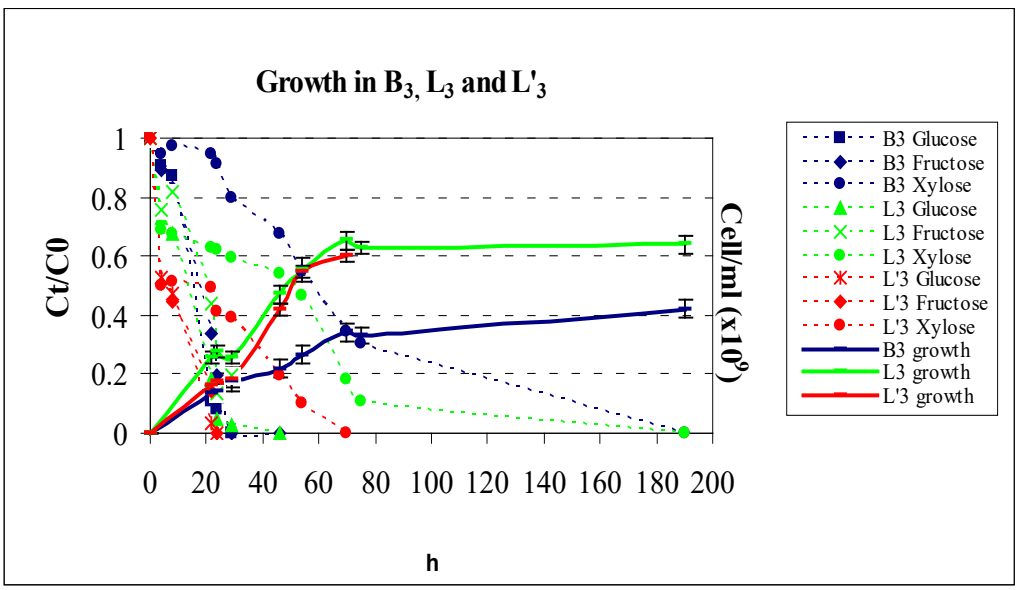
a)


Growth in B1, L1 and L'1
Ct/C0
Cell/ml (x10⁹)
h
B1 Glucose
B1 Fructose
L1 Glucose
L1 Fructose
L'1 Glucose
L'1 Fructose
B1 growth
L1 growth
L'1 growth

b)


Growth in B2, L2 and L'2
Ct/C0
Cell/ml (x10⁹)
h
B2 Glucose
B2 Fructose
L2 Glucose
L2 Fructose
L'2 Glucose
L'2 Fructose
B2 growth
L2 growth
L'2 growth

c)


Growth in B3, L3 and L'3
Ct/C0
Cell/ml (x10⁹)
h
B3 Glucose
B3 Fructose
B3 Xylose
L3 Glucose
L3 Fructose
L3 Xylose
L'3 Glucose
L'3 Fructose
L'3 Xylose
B3 growth
L3 growth
L'3 growth