UNIVERSIDADE DA CORUÑA

Master in Molecular and Cellular Biology and Genetics

Bioethanol and recombinant proteins production from milk whey and molasses

Produción de Bioetanol e proteínas recombinantes a partir de soro de leite e molasas

Producción de Bioetanol y proteínas recombinantes a partir de suero de leche y melazas

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D. Manuel Becerra Fernández autorizo a presentación do Traballo de Fin de Máster: **Produción de Bioetanol e proteínas recombinantes a partir de soro de leite e molasas** presentado pola alumna Dña. **Aghayeva Gulshan** estudante do Máster en Bioloxía Molecular, Celular e Xenética para a súa defensa ante o tribunal cualificador.

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Asdo.: Manuel Becerra Fernández

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Introduction

The growing use of fermentation processes to manufacture specific products, including diverse heterologous proteins, enzymes or pharmaceuticals, is creating an increased demand for efficient systems for the recovery of intracellular materials from microorganisms (Becerra *et al.,* 2001). Today, bacteria and yeast are widely applied in the fermentation industry to produce value-added products from renewable resources.

The realization that fossil fuel reserves are limited and that their use pollutes the environment has pushed research for new alternative energy source (Huang, W and Tang, I, 2007). An eco-friendly bioethanol is one such alternate fuel that can be used in unmodified petrol engines with current fueling infrastructure and it is easily applicable in present day combustion engine, as mixing with gasoline (Hansen *et al.*, 2005). Bioethanol is a clean and sustainable alternative to petroleum. It has lower toxicity and is easily biodegradable, soluble in water, harmless to the environment and does not generate greenhouse gases.

World-wide interest in the production of fermentation ethanol (or bioethanol) as a source of renewable energy has oscillated during most of this century. Production was initiated in the 1930s but waned due to low oil prices. During the 1970s oil crisis interest was renewed but subsequently declined after the crisis. In the 1990s, bioethanol attracted more attention due to its application as an octane enhancer and replacer in petroleum (gasoline) for internal combustion engines (Graeme, 1998).

The production of bioethanol in the development of first generation of renewable energy commonly used biomass containing sugar and starch crops as raw materials. The biomass such as sugar cane and cassava which is used in the production of bioethanol, is still categorized as primary food. Since the first generation of new and renewable energy is proofed interfering the food security, the development of second generation of new and renewable energy emphasized on biomass from waste materials such as agricultural waste, and cheese industry waste (whey) (Ariyanti, D. *et al.*, 2012).

Ethanol has tremendous applications in chemistry, pharmaceutical and food industries as a form of raw materials, solvents and fuel. Ethanol production worldwide in 2011 reached 23.4 billion U.S. gallons, where 80% of that produced by fermentation (Ariyanti, D. *et al.*, 2012).

Kluyveromyces lactis is both scientifically and biotechnologically one of the most important non-*Saccharomyces* yeasts. Its biotechnological significance builds on its history of safe use in the food industry and its well-known ability to produce enzymes. *K. lactis* has been used as a source of lactase (β-galactosidase), an enzyme that degrades milk sugar (lactose) and is necessary for production of lactose-free dairy products (Albert J.J. van Ooye, *et al.*, 2006). β-galactosidase has attracted the attention of researches and industries because of its important applications in the fields of medicine (treatment of lactose intolerance), food technology (to prevent lactose crystallization and increase its sweetening power) and the environment (milk whey utilization) (Pereira-Rodríguez, A., *et al.*, 2012).

Saccharomyces cerevisiae is often used in commercial biotechnological processes. However, it cannot ferment lactose since it lacks both β-galactosidase, to hydrolyze lactose to monosaccharides, and a lactose permease system, to transport the disaccharide into the cytoplasm (Becerra *et al.*, 2002). New recombinant *S. cerevisiae* strains in which β-galactosidase from *K. lactis* is secreted have been constructed (Becerra *et al.*, 2001b). Therefore, lactose utilization by these strains could be improved in comparison to strains expressing an intracellular β-galactosidase (Becerra *et al.*, 2002).

In this work, a small research project on the bioethanol and heterologous proteins production by yeasts has been performed. Specifically employed were two strains of *Kluyveromyces lactis* available at the Biochemistry and Molecular Biology Laboratory of the UDC: GG799 (wild strain) and GG799 with *MEL1* integrated in its genome.

MEL1 codes for the α-galactosidase from *S. cerevisiae*. The α-galactosidase catalyzes the hydrolysis of α-D-galactosidic linkages present in galacto-oligosaccharides and polymeric galacto-mannans as well as glucids like stachiose, raffinose and melibiose. Its activity is of interest in many biotechnological applications such as raffinose and other galacto-oligosaccharide hydrolysis in sugar beet industry and in the processing of soybean products and other legume derived food (Fernández-Leiro, R. *et al.*, 2010).

Both strains, GG799 and GG799MEL1, have been grown in milk whey (from a cheese industry and very pollutant for the environment) or molasses (effluent obtained in the preparation of sugar by repeated crystallization, it is a complex mixture of different

sugars, being its main component sucrose) (Fajardo, ES & Sarmiento , SC, 2007) or a mixture of milk whey and molasses.

Milk Whey

Whey, the liquid remaining after milk fat and casein have been separated from whole milk, is one of the major disposal problems of the dairy industry, and demands simple and economical solutions (Koller *et al.*, 2012). To make 1 kg of cheese, 9 kg of whey is discarded to the environment. Whey contains a huge organic material with the value of BOD and COD respectively 50 and 80 g/L (Ariyanti, D. *et al.,* 2012). Whey represents about 85–95% of the milk volume and retains 55% of milk nutrients. Among the most abundant of these nutrients are lactose (4.5– 5% w/v), soluble proteins (0.6–0.8% w/v), lipids (0.4–0.5% w/v) and mineral salts (8–10% of dried extract).

Whey represents an important environmental problem because of the high volumes produced and its high organic matter content. The search for a satisfactory solution for disposal of the unutilized whey produced in the manufacture of cheese remains an area of intense concern for the dairy industry. Since conventional waste treatment systems are costly, the ideal solution would entail converting the lactose to a marketable product to defray the operating costs and possibly recover the initial capital outlay. One alternative often proposed is to ferment the lactose into ethanol for use as a fuel or chemical feedstock. High ethanol productivity and reduced energy demand have been two important aspects of most alcoholic fermentation research. To achieve them several techniques have been developed including continuous culture, cell immobilization, cell recycle through sedimentation or membrane retention and two stage reactor systems (Koller *et al.*, 2012).

Lactose can be converted into bioethanol through fermentation process using yeast, especially species of *Kluyveromyces*. The presence of lactose in whey as the sole carbohydrate can limit the growth of other microorganisms. This means that *Kluyveromyces* species can be optimally break down the lactose using β-galactosidase enzymes. Beside lactose, whey also contains vitamins and minerals that can improve the physiological activity of cells (Ariyanti, D. *et al.,* 2012).

Molasses

Molasses is commonly used as a feedstock for bioethanol production. Molasses, the non crystallisable residue remaining after sucrose purification, has some advantages: it is a relatively inexpensive raw material, readily available, it does not require starch hydrolysis and already used for ethanol production.

Beet Molasses is a by-product of the manufacture of sucrose from sugar beets. It must contain not less than 48% total sugars expressed as invert and its density determined by double dilution must not be less than 79.50 Brix. IFN 4-30-289 Beet sugar molasses (Dr. Leo V. Curtin, 1983).

The molasses obtained after sugar beet processing contains about 60% sucrose and 40% other components. The non sucrose substances include inorganic salts, raffinose, organic acids and nitrogen containing compounds. Molasses is used in the baker's yeast production, in the fermentation technology for ethanol, citric, lactic and gluconic acids production, as well as glycerol, butanol and acetone production, as an ingredient of mixed feeds or in the production of amino acids (Belitz *et al*., 2009; Satyanarayana *et al.*, 2009).

Cane Molasses is a by-product of the manufacture or refining of sucrose from sugar cane. It must not contain less than 46% total sugars expressed as invert. If its moisture content exceeds 27%, its density determined by double dilution must not be less than 79.50 Brix. IFN 4-13-251 Sugar cane molasses (Dr. Leo V. Curtin, 1983).

OBJECTIVES

The main objectives of this work were:

Evaluation of two strains of *Kluyveromyces lactis*: wild type GG799 and one variant thereof, the GG799MEL1 containing the *MEL1* gene coding for the α-galactosidase from *Saccharomyces cerevisiae* integrated into its genome, growing in milk whey, milk whey with yeast extract, molasses media (sugar cane and beet) and a mixture of milk whey with molasses medium for:

1. Biomass production (cell growth) in different media.

2. Ethanol production in different media.

3. Production of enzymes of industrial interest: α-galactosidase and β-galactosidase.

4. Sugar consumption in different media.

Material and methods

3.1 Strains used

It has used the wild strain of *Kluyveromyces lactis* GG799 (New England Biolabs) and GG799MEL1 strain obtained by genetic engineering techniques in the Biochemistry and Molecular Biology Laboratory at the University of A Coruña. The strain GG799MEL1 presents several tandem copies of the *MEL1* gene encoding the α-galactosidase from *Saccharomyces cerevisiae*, integrated in the genome of the wild type GG799 in the promoter region of the *LAC4* (gene encoding the *K. lactis* β – galactosidasasa).

The *K. lactis* Protein Expression vector (pKLAC1) contains a variant of the strong *K. lactis LAC4* promoter (PLAC4-PBI) for expression of a desired gene in *K. lactis*. The major advantage of the PLAC4-PBI promoter is that it is transcriptionally silent in *Escherichia coli*. In contrast, the wild-type PLAC4 promoter shows background transcriptional activity in *E. coli* which can be detrimental to the process of assembling or amplifying expression constructs in *E. coli* prior to their introduction into yeast cells. This is especially problematic if the cloned gene of interest encodes a translated product that is toxic to *E. coli* cells. Therefore, pKLAC1 is well-suited for the cloning and yeast expression of genes encoding proteins that are toxic or otherwise detrimental to bacteria (Guimarães *et al.*,2008). The promoter region of the gene *LAC4* is reconstituted after integration. The strain GG799MEL1 thus constructed is capable of expressing under the control of the *LAC4* promoter, inducible by galactose and repressed by glucose, the recombinant protein α -galactosidase as well as the native β-galactosidase protein. The recombinant α -galactosidase protein is directed to the secretory pathway by the secretion signal of the α - mating of *S. cerevisiae*, so that a percentage of the activity of this protein is detected in the extracellular medium. While the protein is being processed and thus has not yet reached the extracellular medium, it can be detected inside the cells. The native β -galactosidase protein is an intracellular protein for which the activity is measured intracellularly. Both strains, GG799MEL1 and GG799, secrete the native protein invertase into the extracellular medium, which is encoded by the gene *INV1* (Flórez, 2013).

3.2 Culture medium and conditions

The strains were grown using milk whey, molasses (from sugar cane and beet) and also a medium with milk whey and molasses.

Milk whey

The permeate concentrated from ultrafiltered milk whey was ceded by the cheese factory QUEIZUAR, SL (Bama, A Coruña). Once brought from the factory, was stored frozen at -20° C in aliquots liters. As a preliminary step to use immediately after thawing was sterilized in autoclave (121 ° C for 20 minutes) resulting in coagulation of the majority of proteins. To separate such proteins, the serum was centrifuged under sterile conditions (15 min at 10,000 rpm). The supernatant thus obtained was used as culture medium.

Molasses.

Molasses from sugar cane and beet were provided by the sugar company AZUCARERA EBRO (AB Sugar Group, Spain). Previous their use, they were diluted 2-times and were supplemented with salts: $(NH_4)_2SO_4$ 1g/L , $MgSO_4 \cdot 7H_2O$ 0.5 g/L and KH_2PO_4 1 g/L and autoclaved.

Cultures were performed by duplicate in two flasks (for each strain) with 20 mL of cultivation at 30 ° C in orbital shaking at 250rpm. Samples were taken periodically to determine growth, enzyme activity, ethanol production and sugar consumption: lactose in the case of whey and glucose in the case of molasses.

At each sampling, 1mL of culture was centrifuged (5000 rpm for 5 minutes) and the supernatant spreading it into two aliquots of 500 μL in eppendorff tubes. One was stored at 4 ° C in the refrigerator for measuring activity of α - galactosidase and extracellular enzymes. The other was stored at -20 ° C freezer for measuring ethanol production and sugar consumption. The cell pellet was stored in the freezer at -20 ° C for measurement of α and β - galactosidase intracellular activity (Flórez, 2013).

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3.3 Measurements of enzyme activity

3.3.1 *Determination of intracellular and extracellular α -galactosidase activity with PNPG (p -nitrophenyl- α -D- galactopyranoside) .*

The activity of α – galactosidas *in vitro* was carried out using the chromogenic substrate p -nitrophenyl - α -D- galactopyranoside (PNPG) (Sigma) following the method described by Ryan *et al* (Ryan *et al.*, 1998) . The PNPG colourless compound results in a product after hydrolysis which, because of the change in pH produced by the stop reaction buffer acquires a measurable yellow colour using a spectrophotometer and it is proportional to the amount of substrate released.

To carry out intracellular activity measures 250 µl of yeast culture was decanted by centrifugation and resuspended in 250 µL of reaction buffer (20 mMHepes pH 7.5, 0.002% SDS and 10 mM DTT) ; 30 µL of chloroform was added ; aliquots were shaken vigorously to permeabilize the cells, and incubated 10 minutes at 40° C. For the determination of the extracellular activity, variable volumes were completed with the reaction buffer (61mM citric acid and 77 mM Na_2HPO_4 pH 4) until 110 µL and incubated 10 minutes at 40 ° C. After incubation at 40 ° C , required for the sample to reach the temperature optimum, 110 µL of PNPG 10 mM was added (prepared in the following reaction buffer: citric acid 61 mM Na_2HPO_4 77 mM pH 4) and aliquots of 100 µL were collected at different times (usually 2 to 4 minutes) . To stop the reaction 100 µL of the reaction was added to an ependorff tube with 100 µL of 1M Na_2CO_3. Subsequently absorbance at 400 nm in a plate reader was measured. The α - galactosidase enzyme activity is expressed in enzymatic units; being the enzyme unit (EU) the amount of enzyme which liberates one mol of p -nitrophenol per minute under test conditions (U.E.= µmol x min^{-1} x mL^{-1}). For the calculation, the following formula was used:

$$EU = [\Delta Abs_{400} / \Delta To \times V \times \varepsilon] \times DF = VR \; EU / mL = mol \times min^{-1} \times mL^{-1}$$

Where $\Delta Abs_{400} / \Delta To$ is the increase in absorbance per unit time (min^{-1}) , V is the volume in mL culture and ε is the molar extinction coefficient of the p-nitrofenol at 400 nm (18200 M^{-1} x cm^{-1}) . VR is the total reaction volume in mL and DF realizes the dilution factor applied to the sample for measurement.

3.3.2 β-Galactosidase activity assays

Intracellular β -galactosidase activity was measured by the method of Guarente (1983).

The enzymatic activity of β-Galactosidase is performed by measuring the amount of o-nitrophenol released from o-nitrophenyl- β -D- galactopyranoside (ONPG). The liberated o-nitrophenol turns yellow colour and is quantitatively determined by using a spectophotometr at 410 nm. To measure cell activity, 250 μl of cells were centrifuged 5 minutes at 5000 rpm, the supernatant removed and the precipitate was washed once with Z-buffer (100 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mMKCl , 1.6 mM $MgSO_4$ and 2.7 ml of β - mercaptoethanol per liter of solution, pH 7), then resuspended in 250 μl of the same buffer. After that, it was added 11 μl of chloroform, and 17.5 μl of 0.1% SDS, which act as permeabilizing agents. It was vortexed for 30 seconds and preincubated at 40 ° C for 10 minutes after which 44 μl of substrate solution (4 mg / ml ONPG in distilled water) was added and let the reaction took place, at the same temperature, until the appearance of the characteristic yellow colour of the product (2-4 minutes). From this point, two aliquots of 100 μl at intervals of controlled addition of the substrate were taken. The reaction was stopped by adding 100 μl of these aliquot to a 1 M sodium carbonate solution contained in an Eppendorf tube. The mixture was centrifuged at 13,000 r.p.m. for 5 minutes to remove cells. In the supernatant was determined spectrophotometrically the o-nitrophenol liberated, by measuring absorbance at 420 nm. The concentration is calculated using the following molar extinction coefficient: $4,500 M^{-1} \times cm^{-1}$ (Inchaurrondo*et al*., 1994). The enzyme unit (EU) was defined as the amount of enzyme which liberates one mol of o-nitrophenol per minute in the test conditions. The units are given as EU / ml of culture medium.

## 3.4 Determination of sugars consumption

The determination of the consumption of sugars was performed in milk whey and molasses. The consumption of sugars in whey has been done for the DNS (3, 5-Dinitrosalicilid acid) method but in this case as lactose is a reducing sugar is not necessary to perform any prior hydrolysis to prepare the solution. To the sample was added DNS (in a 1:1). It is necessary to make a standard curve with a lactose solution (6 g / L).

To measure consumption of sugars in molasses the technique of determination of total carbohydrates (phenol sulphuric reaction) has been used, because in molasses there are a variety of sugars, reducing and non-reducing. The determination of total carbohydrates is based on the reaction of the furfural, the applied method (Dubois *et al.*, 1956, as described by Strickland & Parsons, 1968) involves an assessment of the monosaccharides present in the sample to be studied, plus those obtained by acid hydrolysis of the disaccharides. For this, it is necessary to blend our sample (200 µl l of sample or sample dilution) with equal volume of an aqueous solution (50 mg / ml) of phenol and then add 1000 µl l of a solution (5 mg / ml) sulphate Hydrazine (N_2H_4-H_2SO_4) in concentrated sulphuric acid (hydrazine sulphate prevents the spontaneous oxidation of phenol in the reaction mixture, so it is essential to be added quickly). As it is a highly exothermic reaction, the reaction must be cooled at room temperature and the absorbance measured in a spectrophotometer at 490 nm against a blank in which the solution is replaced by distilled water.

The calibration is performed by a series of dilutions prepared from an aqueous solution of glucose. A suitable concentration range is 12-125 mg / ml (Flórez, 2013).

3.5 Determination of the ethanol concentration

For measurement of the ethanol concentration in the extracellular medium, a commercial kit has been used (Nzytech genes and enzymes), in which the amount of ethanol is quantified by a colorimetric method, this method is intended to perform the following reactions:

Ethanol + NAD^+→Acetaldehyde + NADH + H^+ (1)

Acetaldehyde + NAD^+ + H2O + →Acetate+ NADH + H^+ (2)
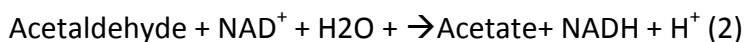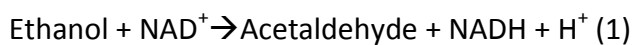
This method is based, therefore, on oxidation, in alkaline conditions, of the ethanol to acetaldehyde in presence of the alcohol dehydrogenase and nicotinamide adenine dinucleotide (NAD). Acetaldehyde formed is then oxidized by the enzyme aldehyde dehydrogenase and NADH generating acetate.

The amounts of NADH formed in the two reactions are in a stoichiometric ratio with respect to the ethanol present in the sample. The increase in NADH is determined by measurement in a spectrophotometer at a wavelength of 340 nm .The determination was

performed following the instructions of the supplier. It should first be carried out the reaction 2 to avoid overestimating the amount of ethanol present in our sample, because acetaldehyde can be derived from cellular metabolism (to the absorbance of the first step has to be subtracted the second measurement). It is also important to avoid contamination, it is advisable to work in areas where there has not been working with ethanol.

Results and discussions

4.1 Growth in milk whey.

The batch kinetics of ethanol production from milk whey was studied in detail. Figure 1 shows growth pattern of *Kluyveromyces lactis* GG799 and GG799MEL1 growing on whey contained 8% lactose. When milk whey is inoculated with the *Kluyveromyces lactis* GG799 or GG799MEL1 strains, the organism selectively take up the dissolved nutrients from the medium and convert them into biomass and ethanol.
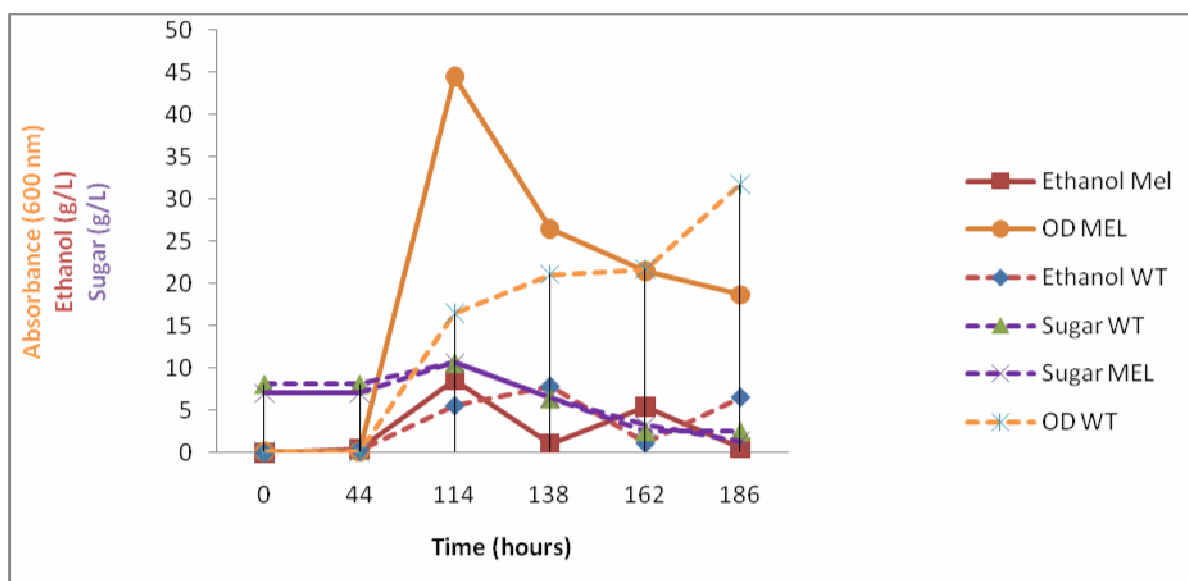
Figure 1: Absorbance (600 nm), ethanol production (g / L) and sugar consumption (g/L) for the wild strain GG799 (dashed line) and GG799MEL1 (solid line) strain growing in concentrated milk whey.

It can be seen in Figure 1 that the genetic modification introduced into the GG799MEL1 strain does not improve the growth in milk whey. Bioethanol production and sugar consumption is almost the same. In order to get better results, we had tried to measure these parameters in milk whey supplemented with yeast extract (1% of the yeast extract).
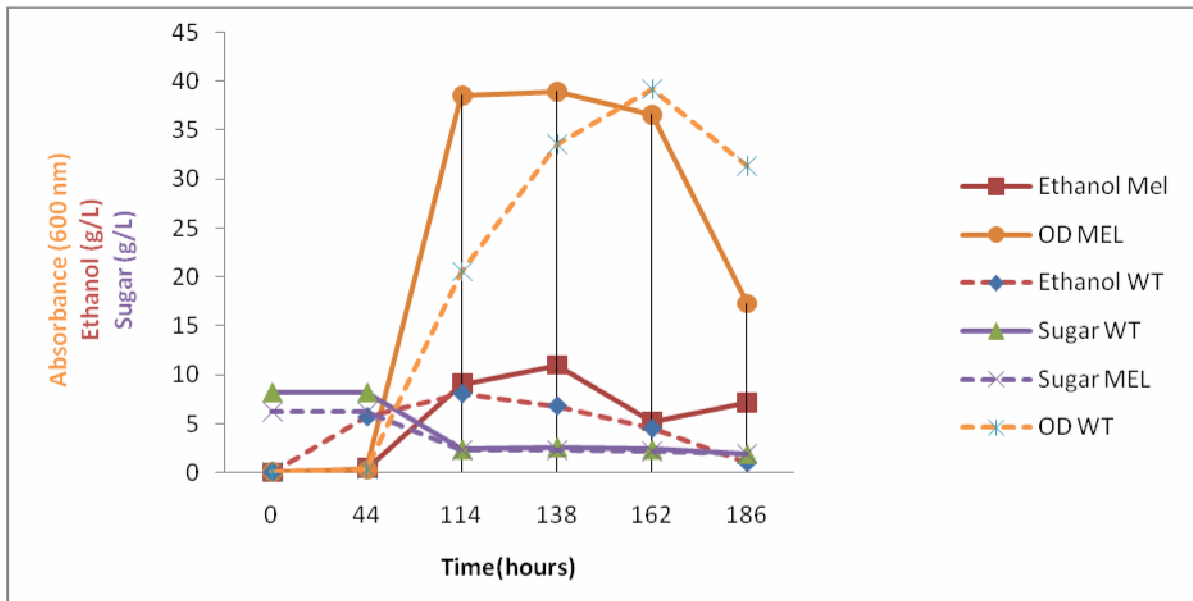
Figure 2: Absorbance (600 nm), ethanol production (g / L) and sugar consumption (g/L) for the wild strain GG799 (dashed line) and GG799MEL1 (solid line) strain growing in concentrated milk whey with 1% of yeast extract.

In this case (Figure 2), the growth and bioethanol production is slightly higher in milk whey with yeast extract than without yeast extract. Although the insertion into the genome of strain GG799 the gene encoding the enzyme α-galactosidase (*MEL1*) does not offer any benefit to degrade lactose, this strain shows a faster growth and bioethanol production than the wild type. The sugar consumption was similar for both strains in both media (Figure 1 and 2).

One of the advantages of the mutant strain is the secretion of α -galactosidase enzyme into the extracellular medium (Figure 3 and 4).In general, the extracellular activity is lower than the intracellular.

After analyse the results and graphs from the milk whey alone and milk whey supplemented with yeast extract (Figure 4 and 5), we can observe that α –galactosidase intracellular and extracellular activity is slightly lower in milk whey than in milk whey with yeast extract. The decline in the production of α -galactosidase in the mutant strain could be due to an increase in the production of the β-galactosidase. β-galactosidase activity in the medium with yeast extract is slightly higher than without.

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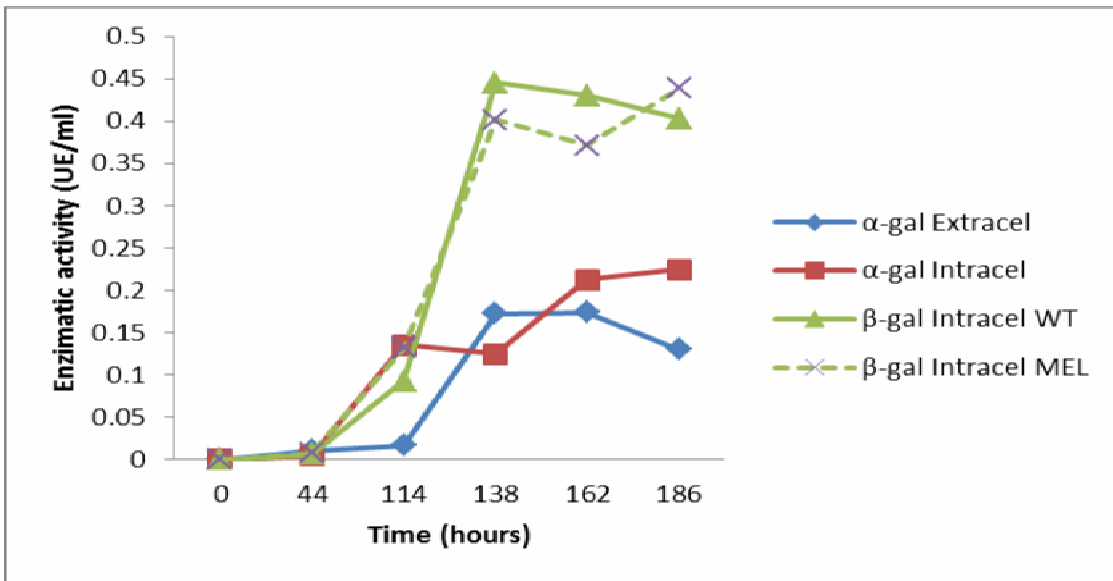
Figure 3: Production of β-galactosidase and α-galactosidase (extracellular and intracellular) for the wild strain GG799 (dashed line) and GG799MEL1 (solid line) strain growing in concentrated milk whey.
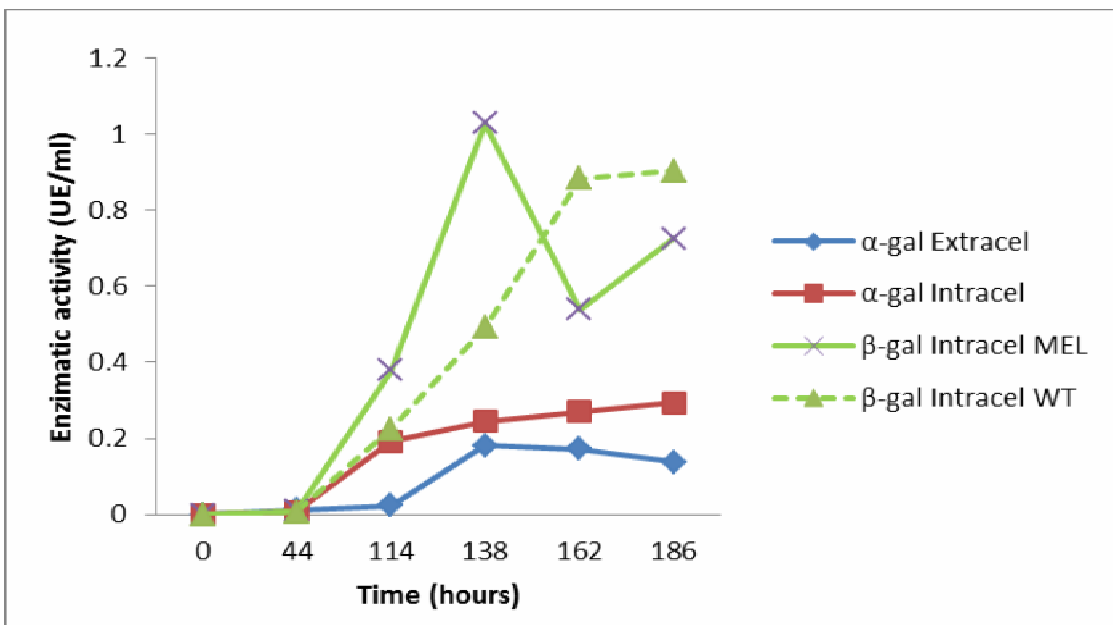

Figure 4: Production of β-galactosidase and α-galactosidase (extracellular and intracellular) for the wild strain GG799 (dashed line) and GG799MEL1 (solid line) strain growing in concentrated milk whey with 1% of yeast extract.

As a conclusion of these experiments, the GG799MEL1 strain, besides of the production of α-galactosidase, has a higher growth and production of intracellular β – galactosidase and also higher ethanol synthesis in milk whey supplemented with yeast

extract than in concentrated milk whey and shows in general a slightly higher growth, bioethanol and β-galactosidase production than the wild strain.

4.2 Growth in molasses media (sugar cane and beet).

In the molasses, a lot of fermentable sugars are found, including sucrose, glucose, fructose and raffinose (Fajardo, EE & Sarmiento , SC, 2007).
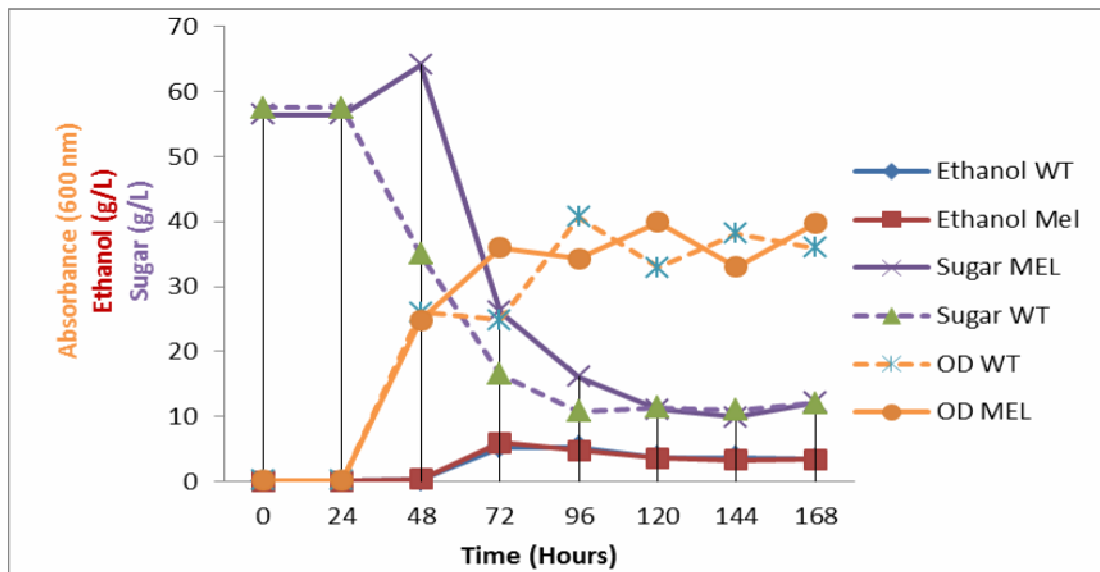

Figure 5: Absorbance (600 nm), ethanol production (g / L) and sugar consumption (g/L) for the wild strain GG799 (dashed line) and GG799MEL1 (solid line) strain growing in sugar cane molasses

Ethanol production in both strains in molasses media (Figure 5 and 6) was lower than in milk whey. In both strains ethanol production is related to the exponential growth, when the cells are growing more rapidly and are therefore favoured the fermentative metabolism. The growth and also the sugar consumption for both strains is faster in sugar cane molasses (Figure 5) than in beet molasses (Figure 6).
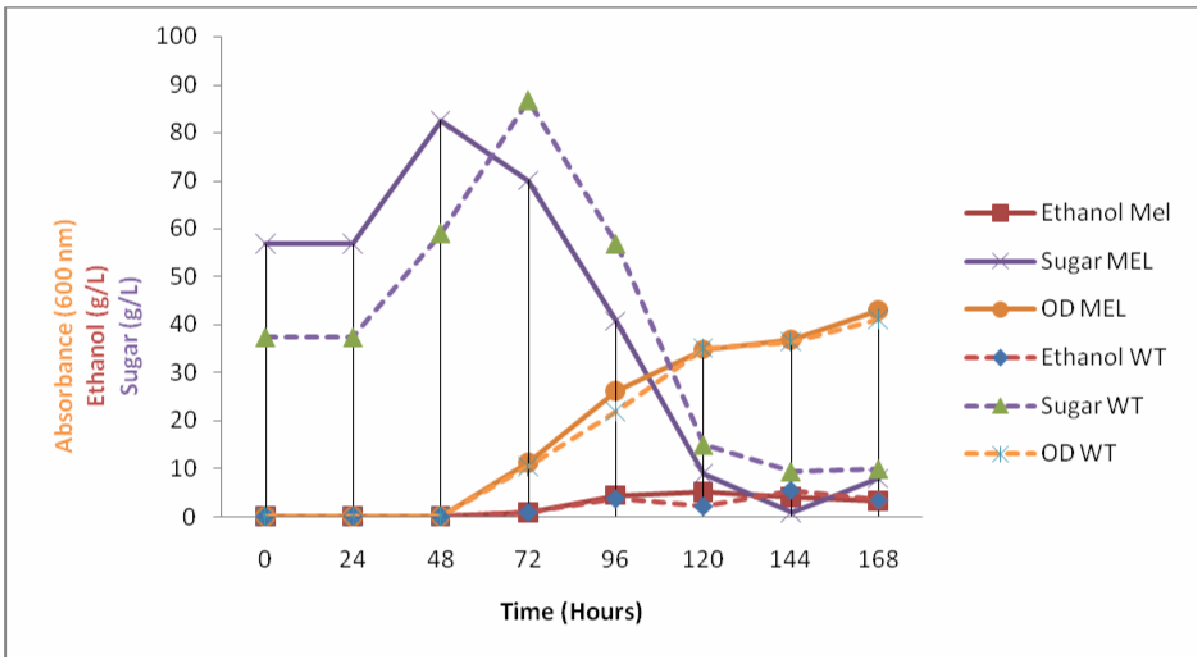
Figure 6: Absorbance (600 nm), ethanol production (g / L) and sugar consumption (g/L) for the wild strain GG799 (dashed line) and GG799MEL1 (solid line) strain growing in beet molasses.

The expression of α - galactosidase is regulated by the lactose-inducible promoter *LAC4* and for that reason no higher expression levels are reached in both type of molasses (Figure 7 and 8).

The wild strain (Figure 7) has a slightly greater β-galactosidase activity than the mutant strain, which is greater if one takes into account the β-galactosidase activity per cell because the growth is slightly lower in the wild strain. Sugar may have been hydrolysed by the invertase and would enable the growth of both strains.

It is remarkable that the β-galactosidase production is higher in sugar cane molasses (Figure 7) than in beet molasses (Figure 8), this result is in accordancewith the growth.
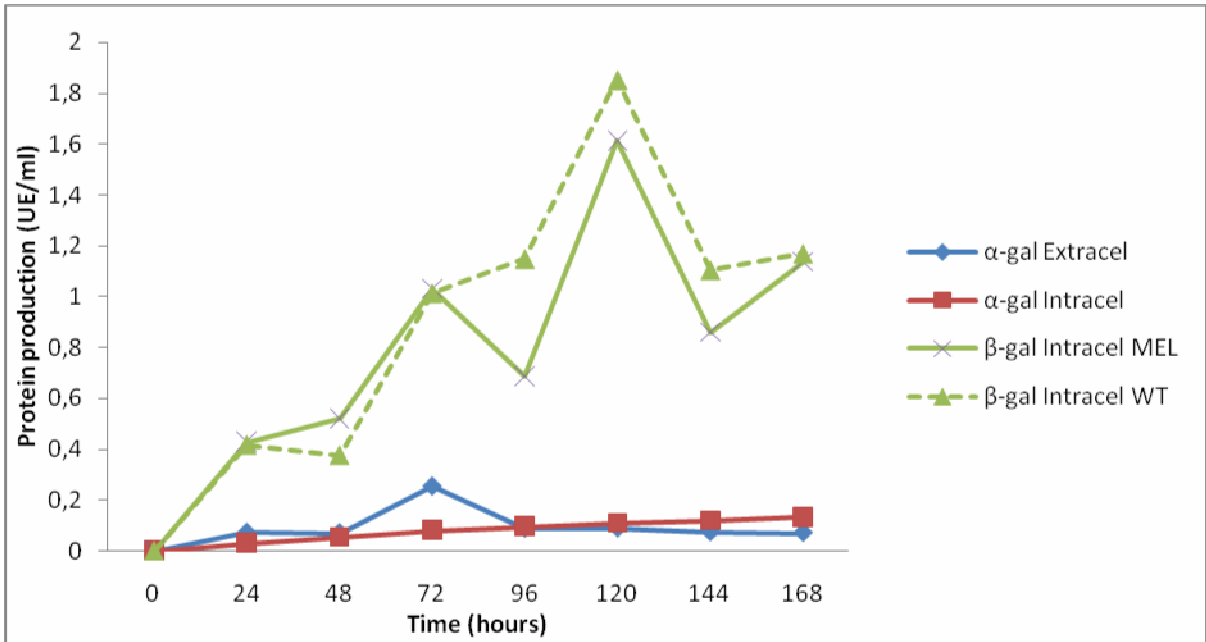
Figure 7: Production of β-galactosidase and α-galactosidase (extracellular and intracellular) for the wild strain GG799 (dashed line) and GG799MEL1 (solid line) strain growing in sugar cane molasses.
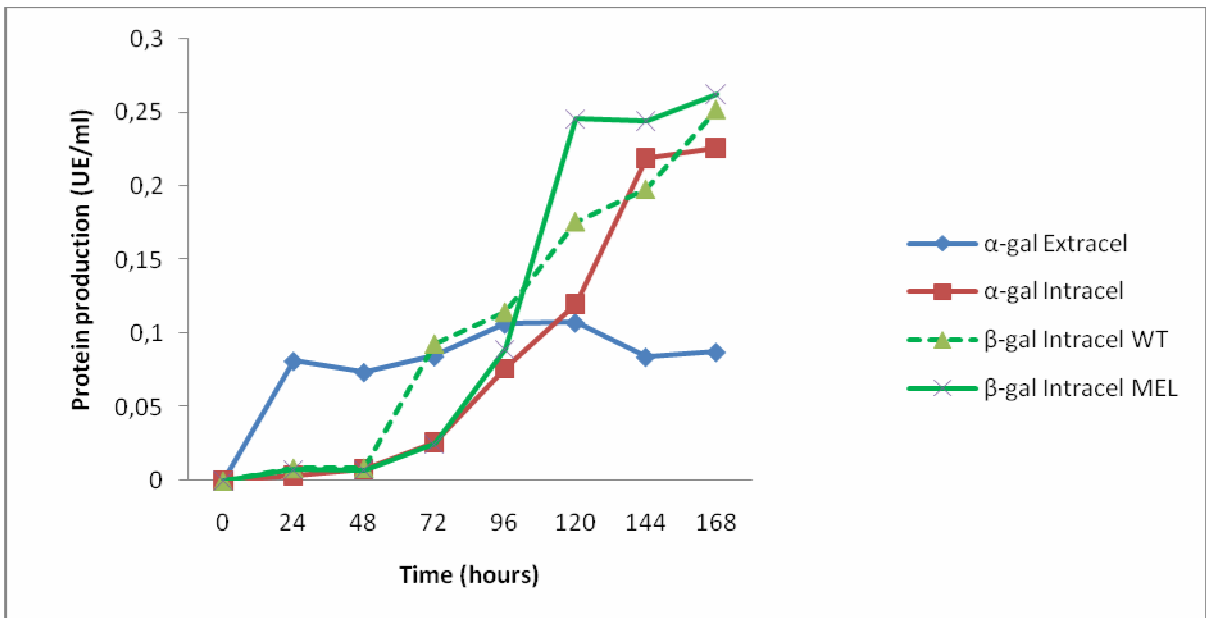

Figure 8: Production of β-galactosidase and α-galactosidase (extracellular and intracellular) for the wild strain GG799 (dashed line) and GG799MEL1 (solid line) strain growing in beet molasses.

4.3 Growth in a mixture of molasses and milk whey (cane molasses and milk whey, beet molasses and milk whey).

After getting the previous results, we had analyzed them:
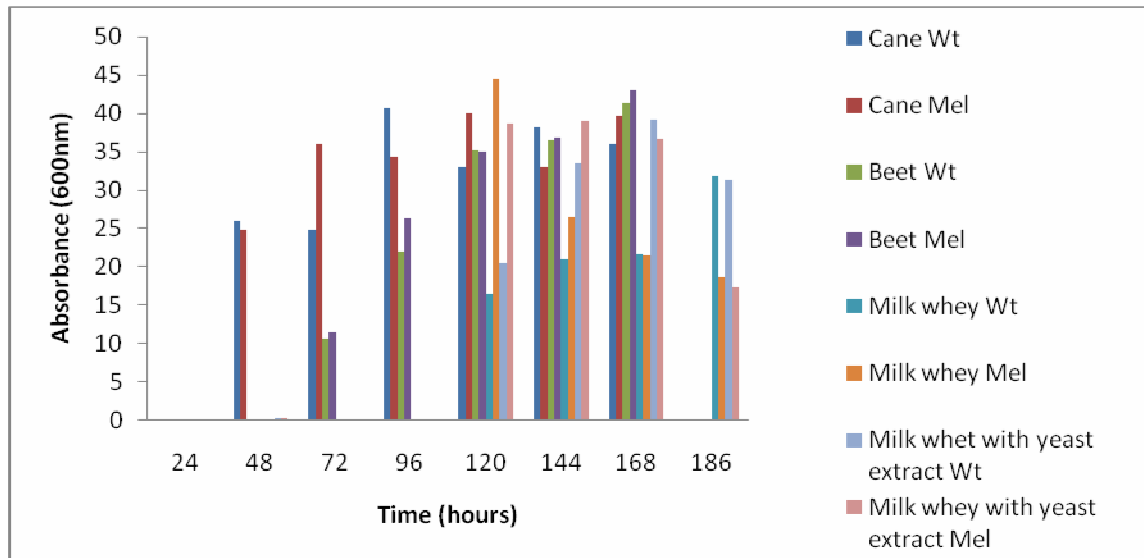
1) The best media to grow is (Figure 9):

Figure 9: Absorbance for the wild strain GG799 and GG799MEL1 strain growing in different media.

The highest absorbance reached is with the mutant strain GG799MEL1 growing in milk whey (Figure 9).

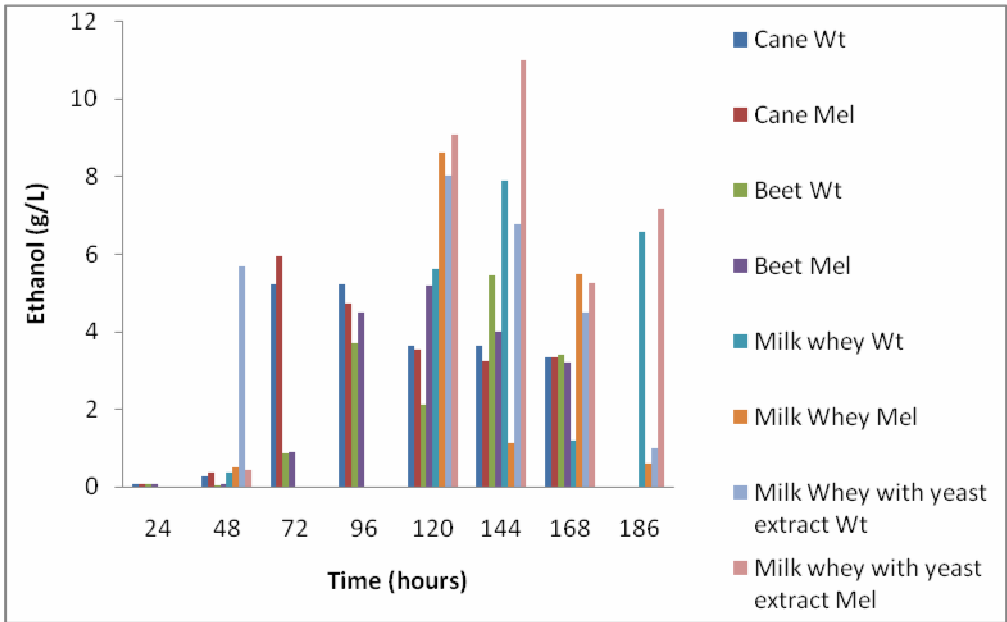2) The best media to reach the highest levels of ethanol is (Figure 10):

Figure 10: Ethanol production (g/L) for the wild strain GG799 and GG799MEL1 strain growing in different media.

The highest ethanol production reached is with the mutant strain GG799MEL1 growing in milk whey supplemented with yeast extract (Figure 10).

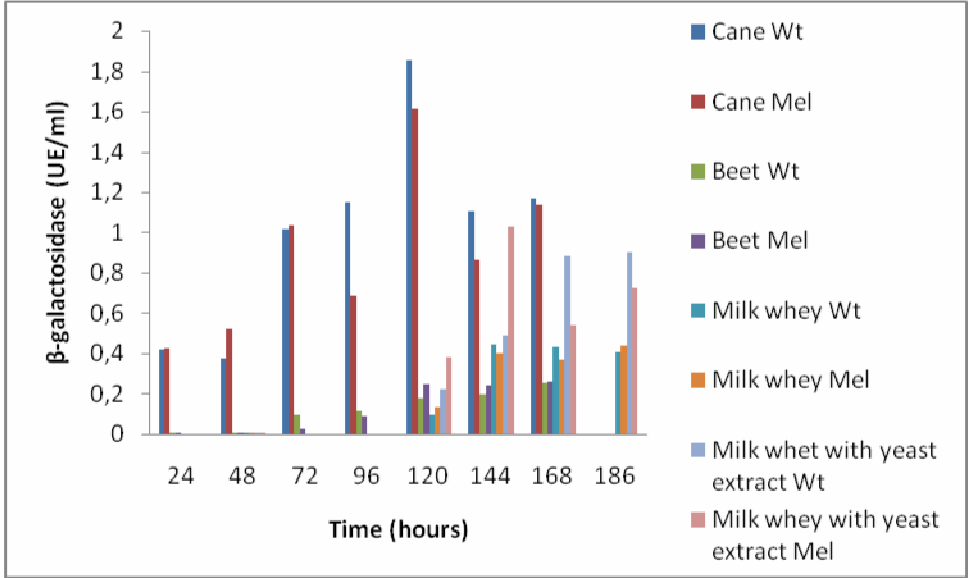3) The best media to reach the highest levels of enzymatic activity (β-galactosidase) is (Figure 11):

Figure 11: β-galactosidase production (UE/ml) for the wild strain GG799 and GG799MEL1 strain growing in different media.

The highest β-galactosidase activity detected is with the wild strain GG799 growing in sugar cane molasses and after that the mutant strain GG799MEL1 in the same medium (Figure 11).

In conclusion, the highest ethanol production medium is milk whey with yeast extract and milk whey (Figure 10) (milk whey wild type=6.58 g/L, milk whey mutant type =8.63, milk whey with yeast extract wild type=8,04, milk whey with yeast extract mutant type=11); sugar cane molasses is the best medium to reach the highest levels of β-galactosidase production (Figure 11) and is a good medium for growing (Figure 9), that is why we have decided to carry out new experiments by mixing milk whey and molasses to determinate the growth, ethanol and enzymatic production of both strains in the mixture media. Both mixture media were prepared to get a final concentration of total sugars of 30%. To get this concentration, it was necessary to mix 50 % of the concentrated milk whey and 13.33 % of the 2-times diluted sugar cane or beet molasses.

The growth (Figure 12) in a mixture of the sugar cane molasses with milk whey is lower than in the other media assayed but it is higher than with beet molasses and milk whey (Figure 13). The ethanol production in a medium with beet molasses and milk whey (Figure 13) is lower than in a medium with sugar cane molasses and milk whey (Figure 12). In the last medium (sugar cane molasses and milk whey) we have the highest ethanol production for the wild strain GG799 (wild type=8.5 g/L, mutant type=7.12 g/L). We can also see a difference in growth between the wild type and mutant in this media; this is probably due to that the mutant strain producing α-galactosidase is able to use more efficiently the sugar from the sugar cane molasses to grow.
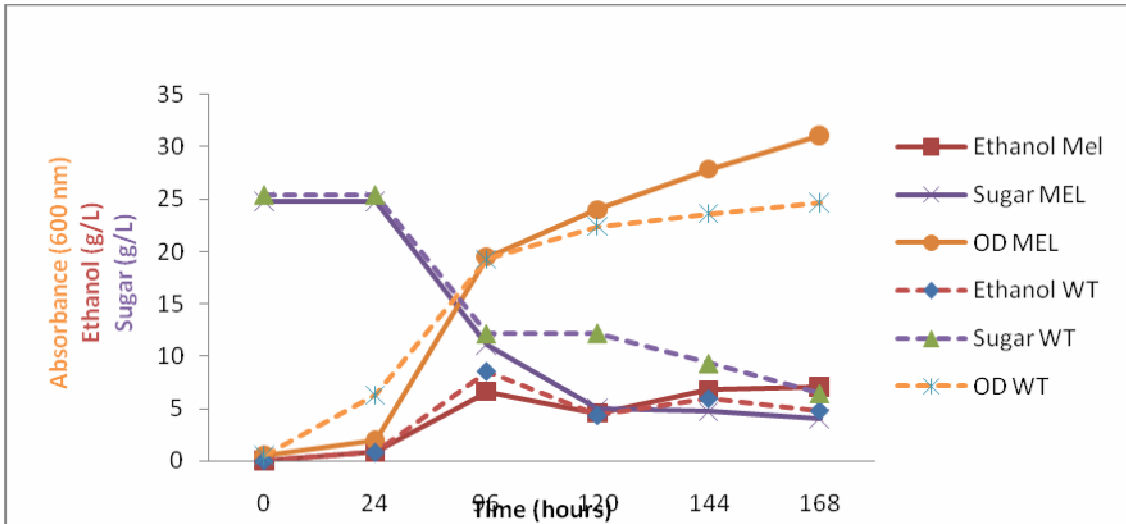
Figure 12: Absorbance (600 nm), ethanol production (g / L) and sugar consumption (g/L) for the wild strain GG799 (dashed line) and GG799MEL1 (solid line) strain growing in a mixture of the sugar cane molasses with milk whey.
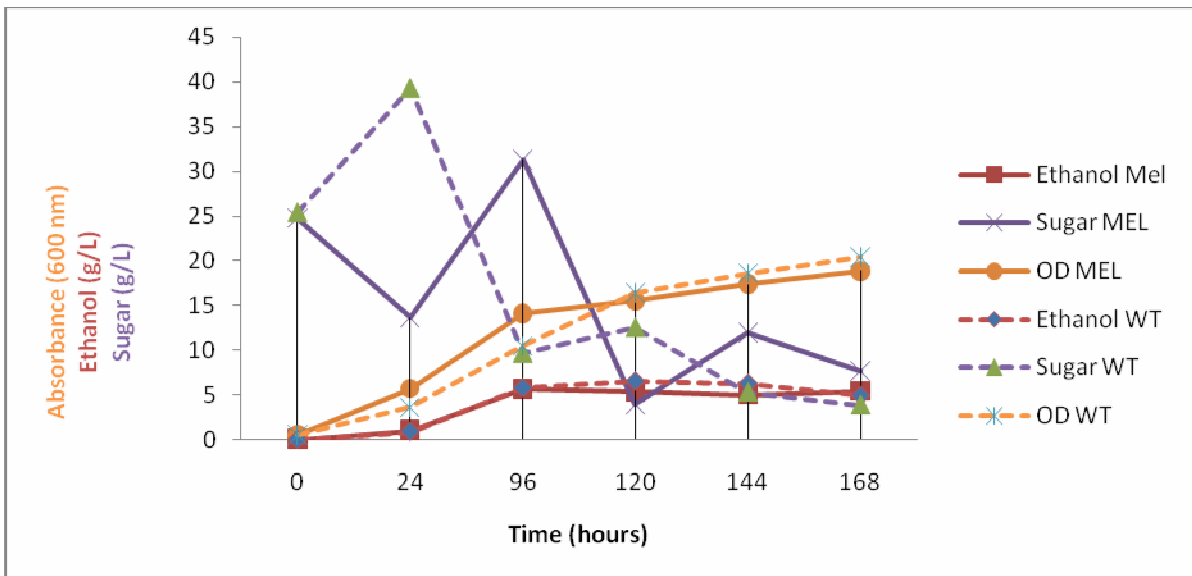

Figure 13: Absorbance (600 nm), ethanol production (g / L) and sugar consumption (g/L) for the wild strain GG799 (dashed line) and GG799MEL1 (solid line) strain growing in mix of the beet molasses with milk whey.
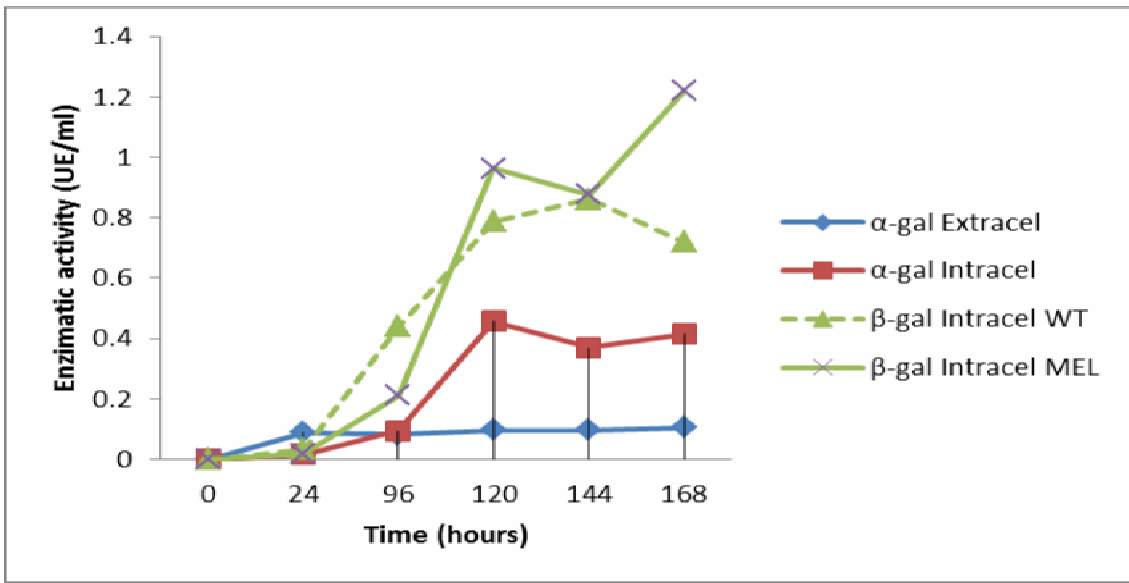
28

Figure 14: Production of β-galactosidase and α-galactosidase (extracellular and intracellular) for the wild strain GG799 (dashed line) and GG799MEL1 (solid line) strain growing in a mixture of the milk whey with sugar cane molasses.
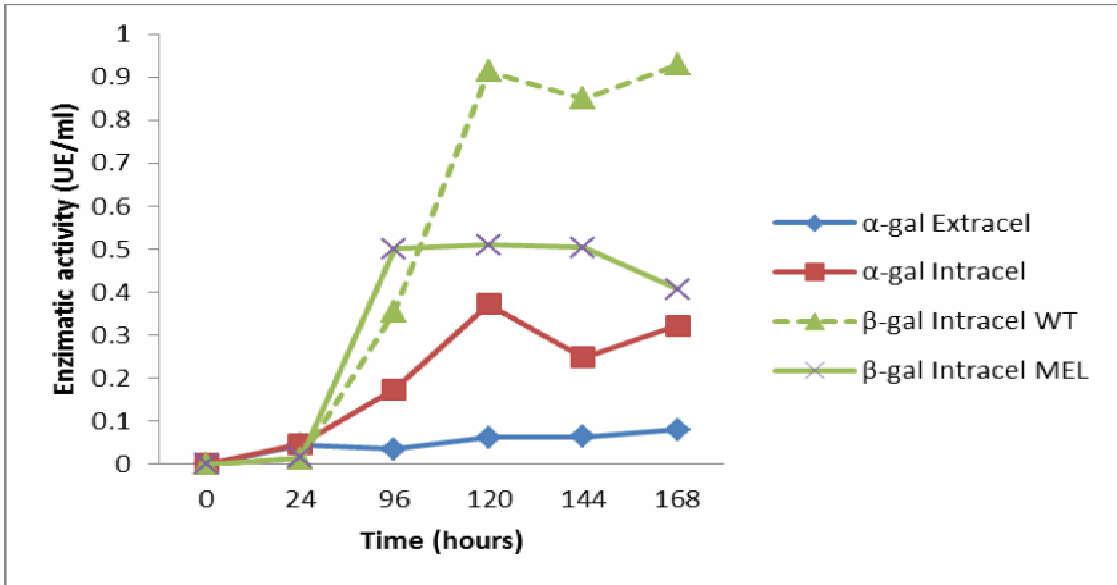

Figure 15: Production of β-galactosidase and α-galactosidase (extracellular and intracellular) for the wild strain GG799 (dashed line) and GG799MEL1 (solid line) strain growing in a mixture of the milk whey and beet molasses.

The β-galactosidase and intracellular α-galactosidase production for the mutant strain GG799MEL1 is higher in sugar cane molasses with milk whey (Figure 14) than in beet molasses and milk whey (Figure 15), however for the wild type strain GG799 the β-

galactosidase production is slightly higher in beet molasses-milk whey than in sugar cane molasses-milk whey. In both medium the intracellular α -galactosidase production is higher than the extracellular activity (Figure 14 and 15).

Conclusions

We can get the following conclusions after comparing all the results:

1) The highest levels of bioethanol production in all the media and strains assayed were reached with the mutant strain GG799MEL1 growing in milk whey supplemented with 1 % of yeast extract (Figure 16).
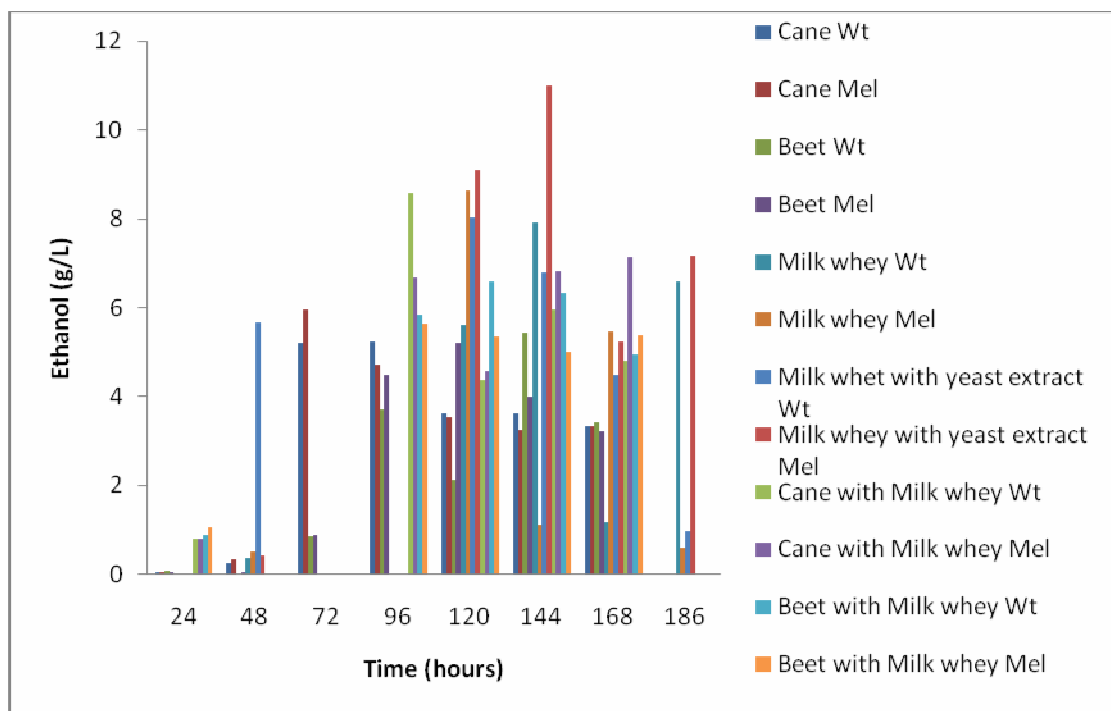

Figure 16: Ethanol production (g/L) for the wild strain GG799 and GG799MEL1 strain growing in different media.

2) The highest levels of β-galactosidase activity detected in all the media and strains assayed were reached with the wild strain GG799 growing in sugar cane molasses (Figure 17). This media was also the best to reach the highest levels of β-galactosidase activity with the mutant strain GG799MEL1.
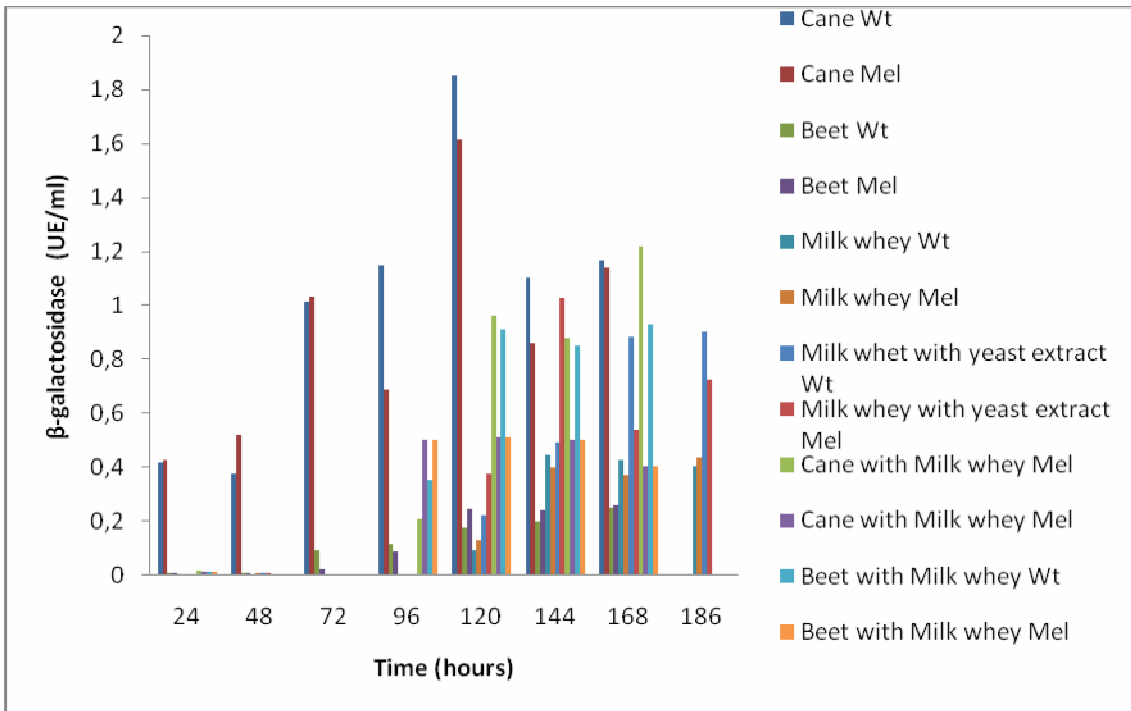
Figure 17: β-galactosidase production (UE/ml) for the wild strain GG799 and GG799MEL1 strain growing in different media.

3) The highest levels of growth determinated in all the media and strains assayed were reached with the mutant strain GG799MEL1 growing in milk whey (Figure 18).
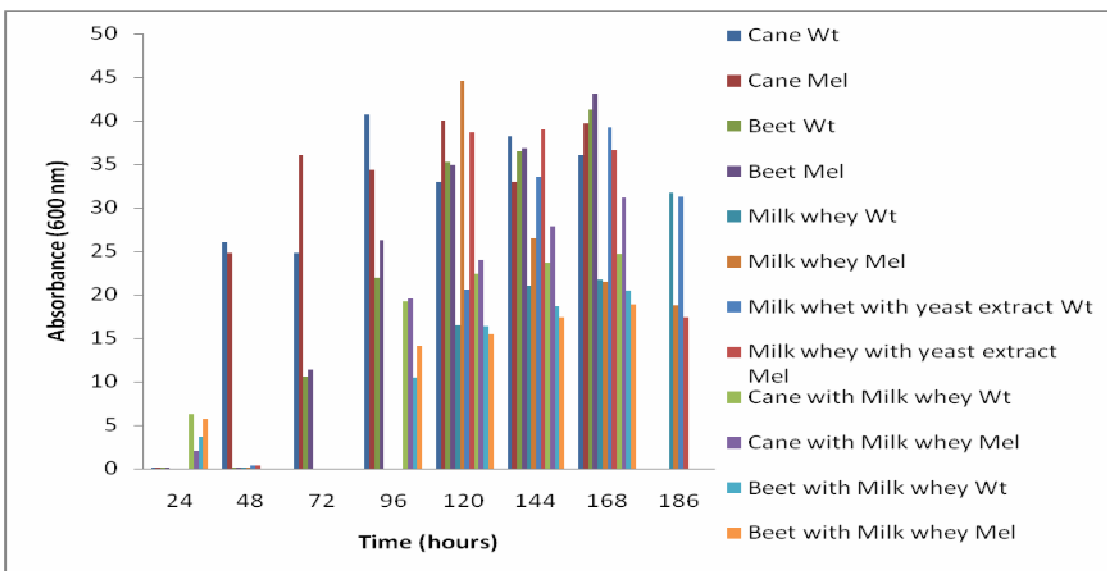

Figure 18: Absorbance (600 nm) for the wild strain GG799 and GG799MEL1 strain growing in different media.

4) The *Kluyveromyces lactis* mutant strain GG799MEL1 is the high biotechnological interest because it can be used to milk whey and molasses valorisation to produce biomass, bioethanol or proteins of interest such as β-galactosidase or α-galactosidase.

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