Bernal, Buenos Aires, 1 de diciembre de 2008

Sra. Paola Bohorquez
Programa de Naciones Unidas para el Desarrollo
Buenos Aires

Estimado Sra.

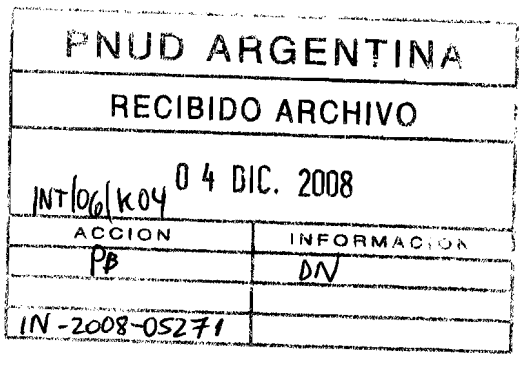
Tengo el agrado de dirigirme a Ud. a efectos de elevar a la PNUD con esta nota, el Informe final del proyecto **"Yeast and yeast derivative production from cheese-whey (YeWHEY)" INT/ 06/K04**.

El informe incluye la descripción de las tareas realizadas en las reuniones inicial y final y el aporte de cada grupo participante con los conocimientos alcanzados como resultado de la cooperación en el marco del proyecto. Se acompaña además un ejemplar del libro "Las levaduras y sus productos derivados como ingredientes en la industria de alimentos", de la editorial Universidad Nacional de Quilmes, del cual somos compiladores y autores, algunos de los participantes del proyecto. En este libro fueron volcados los estudios realizados por nuestros grupos de investigación, muchos de estos estudios finalizados durante el desarrollo del proyecto.

Aprovecho la oportunidad para agradecer a la PNUD, que con este subsidio PGTF hizo posible concretar un proyecto de interés para los países intervinientes y avanzar en el conocimiento y las posibilidades de uso del suero lácteo y de levaduras en la industria de alimentos.

Sin otro particular y a la espera de una evaluación favorable de este informe, la saludo muy cordialmente.

Dr. Jorge R. Wagner
Coordinador del Proyecto



UNITED NATIONS DEVELOPMENT PROGRAM

PEREZ-GUERRERO TRUST FUND FOR ECONOMIC AND TECHNICAL COOPERATION AMONG DEVELOPING COUNTRIES, MEMBERS OF THE GROUP OF 77

PROJECT

YEAST AND YEAST DERIVATIVE PRODUCTION FROM CHEESE WHEY (YEWHEY), Atlas n° 00050046 - INT06/K04

Kick-off meeting report

This meeting was realized in the Universidad Autónoma de Mexico (UAM), city of Mexico D.F. in October 9-10, 2007. The assistants were Dr. Isabel Guerrero Legarreta, Dr. Jorge Soriano and Araceli Tomassini from UAM, Dr. Luis Araya from Universidad de Costa Rica, Eng. Juan Carlos Hidalgo from FIAGRO (El Salvador), Mg. Anahí Cuellas and Dr. Jorge R. Wagner (National University of Quilmes, UNQ, Argentine), and the consultants Lic. Miguel Angel Otero and Eng. Gustavo Saura, from the Cuban Institute for Research on Sugar Cane By-Products (ICIDCA). The participants exposed the problem of whey effluent in dairy industry in Latin American. The different alternatives for the employment of the whey and the incorporation of yeasts and yeast derivatives were analyzed.

Wrap-up meeting report

This meeting was realized in the National Hotel of Habana, Cuba in October 14-17, 2008, in the framework of the Congress Diversification 2008 organized by the Cuban Institute for Research on Sugar Cane By-Products (ICIDCA). The assistants were Eng. Juan Carlos Hidalgo from FIAGRO (El Salvador), Mg. Anahí Cuellas and Dr. Jorge R. Wagner (National University of Quilmes, UNQ, Argentine), and the consultants Lic. Miguel Angel Otero and Eng. Gustavo Saura from ICIDCA. Participants described the advances in the studies planned, which served to prepare the final report. Some members of the project (Dr. Isabel Guerrero Legarreta from

UAM and Dr. Luis Araya from UCR) were unable to attend for professional obligations in their countries, but sent us the results of their studies.

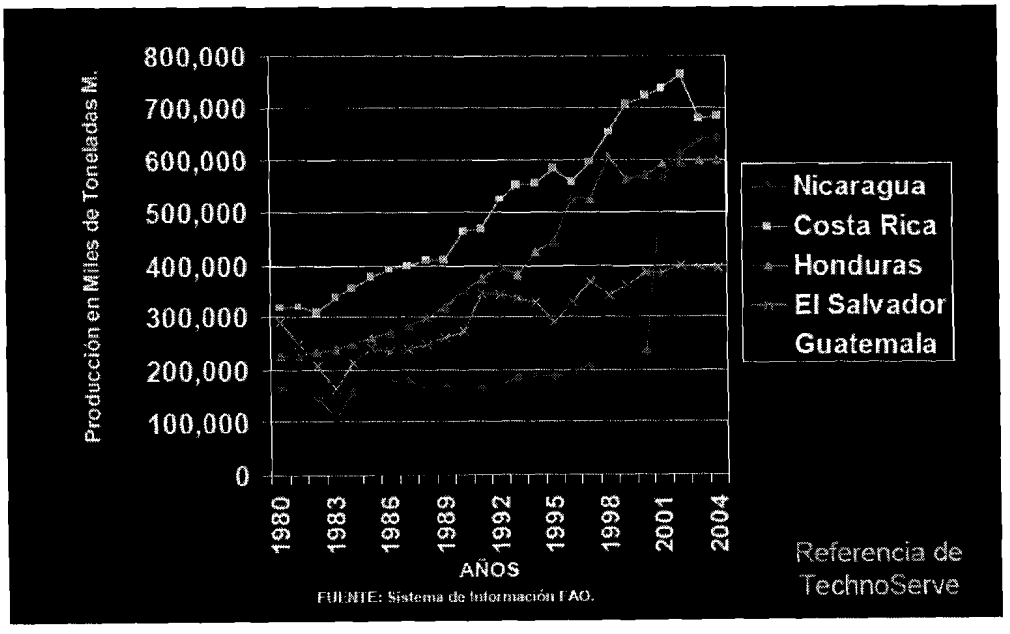
At the congress was presented the book "The yeasts and its derived products as ingredients in the food industry" recently published by National University of Quilmes, in which Jorge Wagner, Miguel A. Otero and Isabel Guerrero were compilers. This publication is the fruitful result of our investigations in yeast, was honored as a result highlights of the Ministry of Sugar in Cuba in 2008. This book is delivered along with this final report.

FINAL REPORT

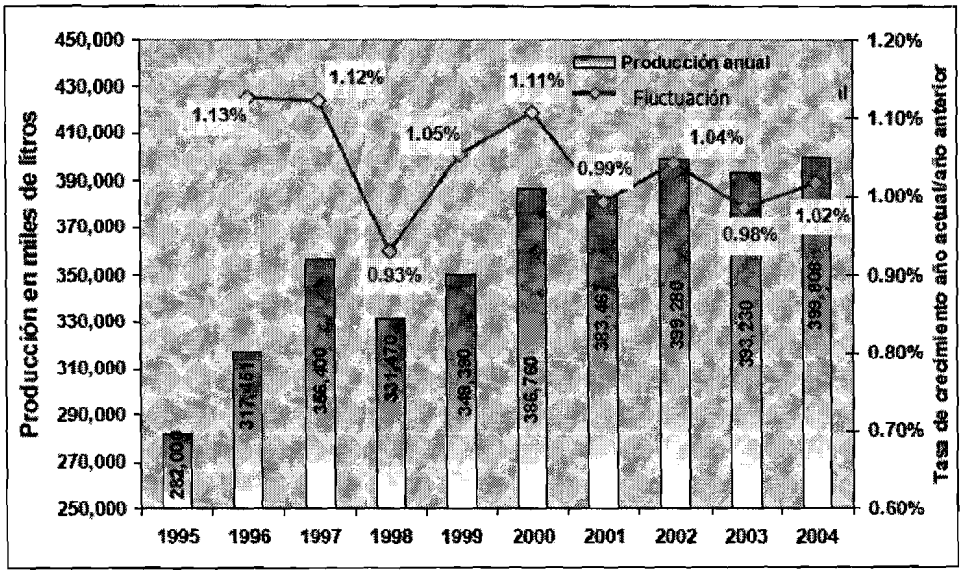
1. POTENTIALITIES FOR THE GOOD USE OF CHEESE WHEY

A) Eng. Juan Carlos Hidalgo - FIAGRO (Foundation for the Innovation in Agricultural Technology, El Salvador)

Estimated production in Central America for the next 25 years



Milk Production from 1995 to 2004 in El Salvador



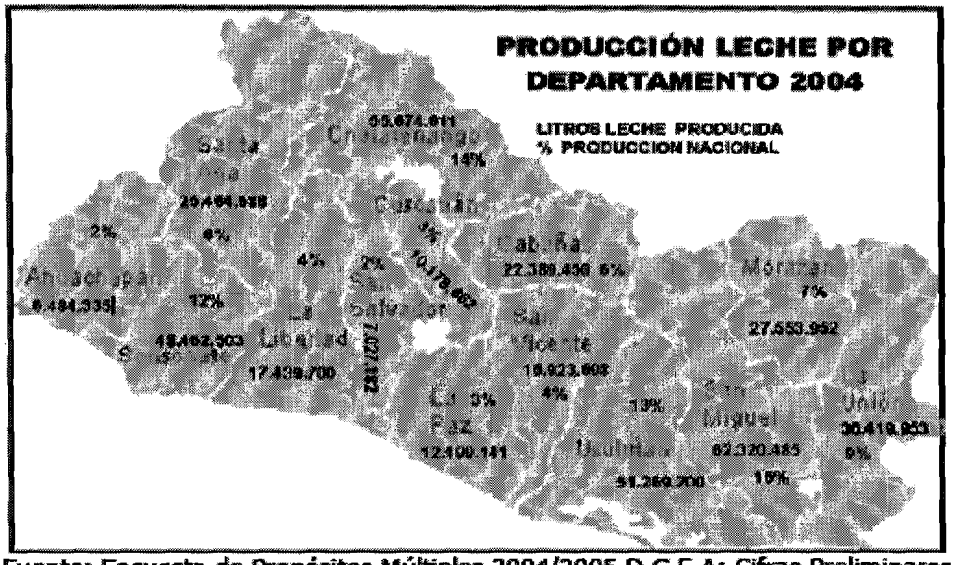
Fuente: Elaborado con base al Anuario Agrícola 2003-2004 MAG, El Salvador.

Distribution of cattle herd

DEPARTAMENTO	TOTAL HEMBRAS	ESTADO REPRODUCTIVO			
		VACAS paridas	VACAS horras	NOVILLAS	TERNERAS
Ahuachapán	**17,327**	4,577	3,672	6,701	2,377
Santa Ana	**58,031**	18,494	14,293	15,944	9,300
Sonsonate	**93,747**	34,206	19,175	24,558	15,808
Chalatenango	**129,707**	41,697	35,336	29,970	22,704
La Libertad	**53,294**	12,643	16,773	16,821	7,057
San Salvador	**20,299**	4,960	5,144	7,665	2,530
Cuscatlán	**29,967**	7,251	6,769	12,246	3,701
La Paz	**22,075**	7,690	3,749	6,998	3,638
Cabañas	**46,301**	15,711	10,340	12,058	8,192
San Vicente	**51,405**	12,284	13,575	20,066	5,480
Usulután	**99,845**	26,977	24,733	29,634	18,501
San Miguel	**173,225**	42,396	42,673	68,126	20,030
Morazán	**68,429**	19,448	19,336	20,582	9,063
La Unión	**64,733**	25,132	10,807	17,348	11,446
TOTAL	**928,385**	**273,466**	**226,375**	**288,717**	**139,827**

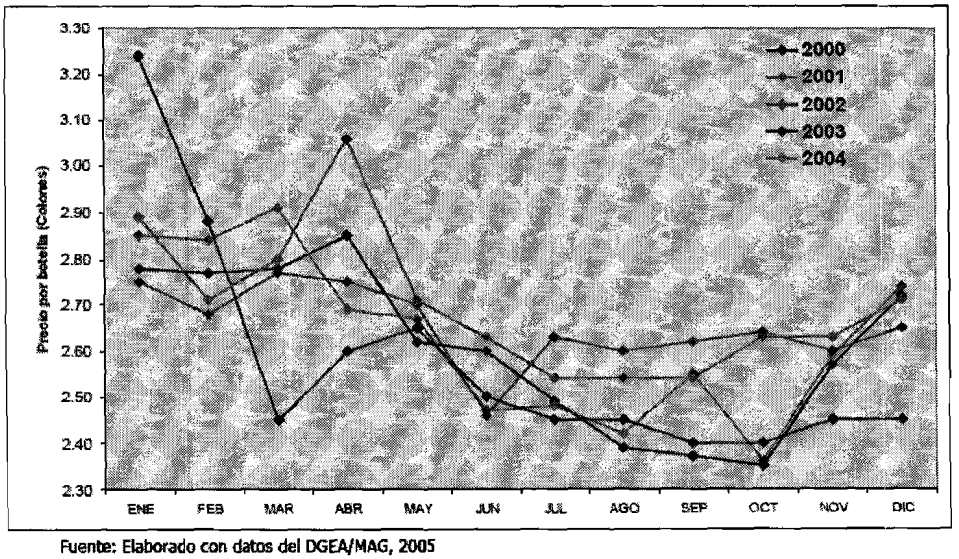
Fuente: Elaborado con datos del DGEA/MAG, 2004

Location of Milk Producers at National Level



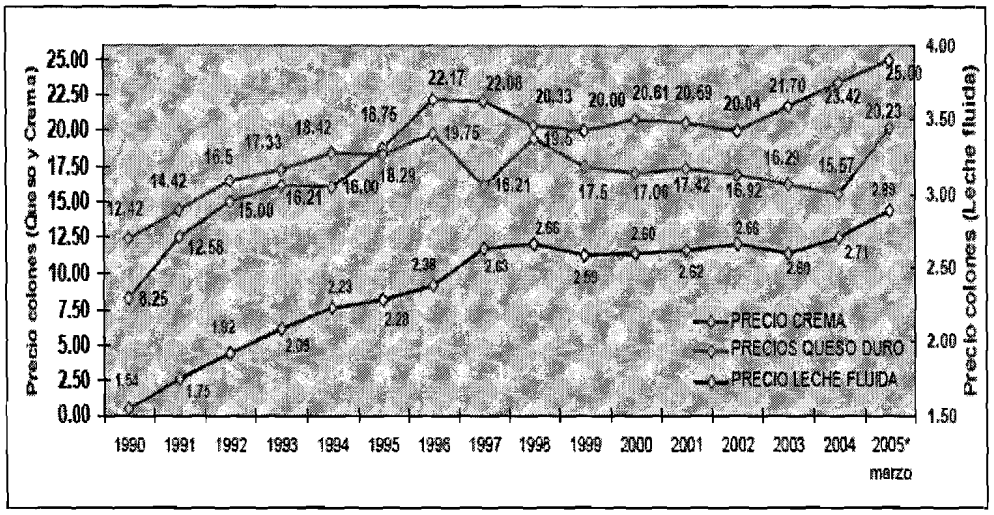
Fuente: Encuesta de Propósitos Múltiples 2004/2005 D.G.E.A: Cifras Preliminares

Prizes to Non-Pasteurized Milk Producers



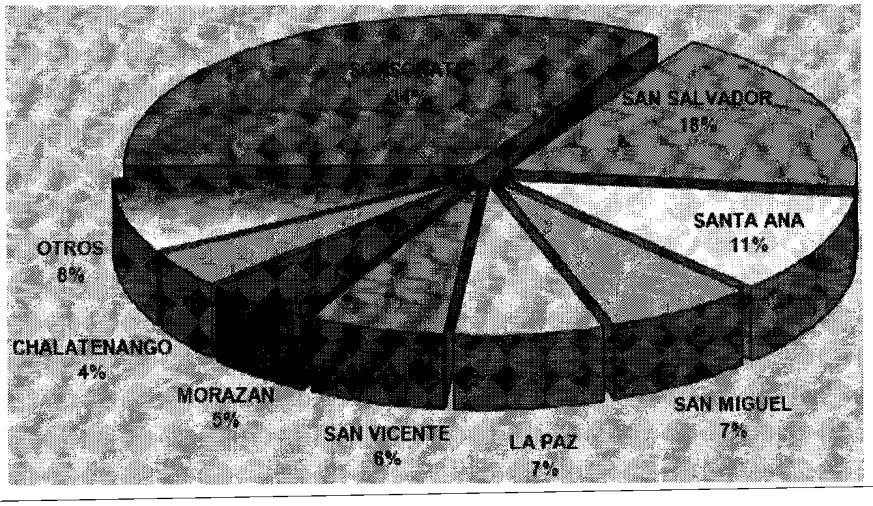
Fuente: Elaborado con datos del DGEA/MAG, 2005

Consumer Prize Evolution 1990-2005



Fuente: Elaborado con datos del DGEA/MAG, 2005

Distribution of Processed Milk Production



Industrial Processers

They process from 7,500 to 37,500 L/day sharing a 49% of the whole processed milk. They are basically six milk industries: Lactosa, Foremost, San Julian, Petacones, La Salud and El Jobo. Each one is specialized in certain products, namely: Foremost and La Salud fluid milk, San Julian and El Jobo in milk creams and Lactosa and Petacones in Cheese.

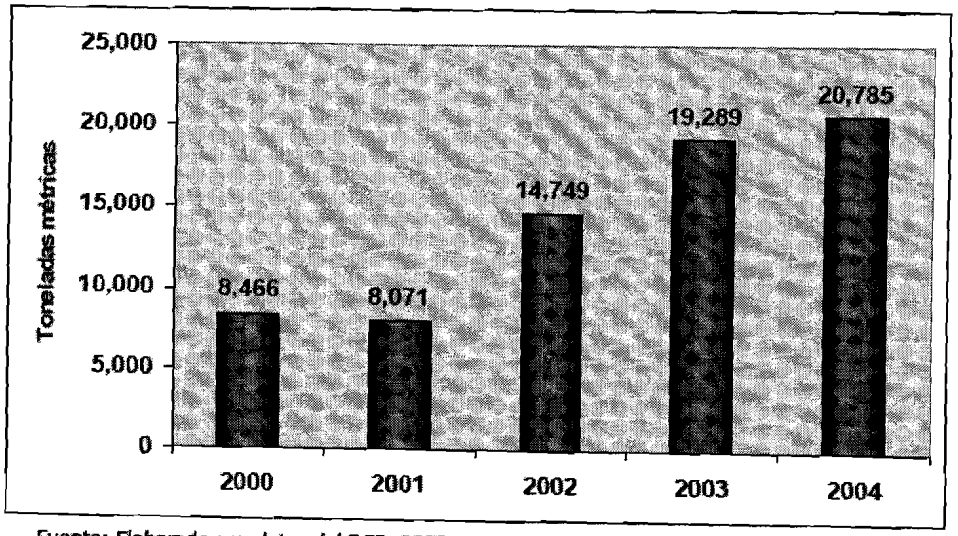
Semi-industrial Processers

Thirty two (32) enterprises at national level that process between 750 and 7,500 L/day, sharing a 19% of the whole milk production. They are specialized in milky products for traditional market, especially as fresh and semi-ripened cheeses.

Artisan Processers

650 units at national level process up to 750 L/day, sharing a 32% of the whole milk market in the country. They are specialized mainly in fresh and cream cheese.

Cheese Imports in El Salvador



Fuente: Elaborado con datos del BCR, 2005

Current whey situation

- Daily milk production: 1,000,000 L (estimated)
- Processed: 700,000 L
- Estimated Liquid Whey: 162 y 288 Millions of Litres/annum (according to different sources)
- Equivalent pollution: 200,655 families
- BOD (Biological Oxygen Demand), COD (Chemical Oxygen Demand) from whey is 100 times higher than average disposal from homes (May 2003, Christian Buser, FHBB)
- Individual cheese consumption is expected to rise.

Conclusions

- There is an enormous potential for the utilization of cheese whey for single-cell protein production
- Present day, cheese whey is an environmental problem affecting enterprise performances
- Dairy sector in El Salvador presents a sustained growth proyection for the next few years.
- Processing industry is well-disposed to use single-cell protein in different applications
- El Salvador has other food industries potentially able to consume single-cell proteins from cheese whey.

B) International Consultants, Biotechnology Division. Cuban Institute for Research on Sugar Cane By-Products (ICIDCA), Havana

INTRODUCTION

Whey is the watery portion of milk remaining after milk coagulation and removal of the curd. Whey can be obtained by acid, heat, and rennet coagulation of milk. There are two types of whey.

Sweet Whey: Sweet whey is manufactured during making of rennet type hard cheese like cheddar or Swiss cheese.

Acid Whey: Acid whey (also known as sour whey) is obtained during making of acid type of cheese such as cottage cheese.

Most of the whey used in different food products is obtained as sweet whey from rennet types of hard cheeses.

Whey is concentrated to obtain sweet/acid whey powder, condensed whey, delactosed powder as the primary products and protein, lactose as secondary products (Ben-Hassan *et al* 1994, Ferrat 1980, Ghaly *et al* 1989).

Fundamentals of Whey Making

The procedure of whey making is similar to cheese making until the curd is formed. It starts with propagating the mother cultures on media and then bulk cultures. The bulk cultures are added to the milk and then rennet preparation is added to milk to form curd in case of sweet whey. The whey is separated and dried.

For acid whey, lactic acid mother cultures are grown on media and then bulk culture are propagated and added to skim milk to make cottage cheese (cottage cheese is also made with the use of rennet). Whey is separated after curd formation and dried as a powder. Table 1 shows an average composition of cheese whey in different presentations.

Sweet whey, a potent pollutant, is produced in large quantities by cheese industries and in most cases is discharged without any treatment to rivers or streams (Ferrat 1980; Ghaly and Singh 1989; Ben-Hassan and Ghaly 1994).

Whey making

Whey deteriorates quickly as a consequence of its rich composition, thus for composition and fermentation studies seems to be more practical to elaborate "whey at home" than obtain it from commercial cheese manufacturers. Though, homemade cheese and therefore its whey could show differences respect to commercial one, composition homogeneity, microbial contaminants and whey deterioration can be held under reasonable limits. This cannot be controlled if commercial whey is used as raw material. Non skimmed powder milk will be used as cheese-milk.

Table 1 Average composition of Sweet and acid Whey

COMPOSITION OF WHEY				
	Fluid Sweet Whey	**Fluid Acid Whey**	**Dried Sweet Whey**	**Dried Acid Whey**
Total Solid	6.35	6.50	96.5	96
Moisture	93.70	93.50	3.5	4
Fat	0.5	0.04	0.8	0.6
Protein	0.80	0.75	13.1	12.5
Lactose	4.85	4.90	75	67.4
Ash	0.50	0.80	7.3	11.8
Lactic Acid	0.05	0.40	0.2	4.2

Milk Treatment

Like most dairy products, cheese-milk must first be clarified, separated and standardized. The milk may then be subjected to a sub-pasteurization treatment of 63-65° C for 15 to 16 sec. This thermal treatment results in a reduction of high initial bacteria counts before storage. It must be followed by proper pasteurization. While HTST pasteurization (72° C for 16 sec) is often used, an alternative heat treatment of 60° C for 16 sec may also be used. This less severe heat treatment is thought to result in a better final flavour cheese by preserving some of the natural flora. If used, the cheese must be stored for 60 days prior to sale, which is similar to the regulations for raw milk cheese.

Homogenization is not usually done for most cheese-milk. It disrupts the fat globules and increases the fat surface area where casein particles adsorb. This results in a soft, weak curd at renneting and increased hydrolytic rancidity.

Inoculation and Milk Ripening

The basis of cheese-making relies on the fermentation of lactose by lactic acid bacteria (LAB). LAB produce lactic acid which lowers the pH and in turn assists coagulation, promotes syneresis, helps prevent spoilage and pathogenic bacteria from growing, contributes to cheese texture, flavour and keeping quality. LAB also produces growth factors which encourages the growth of non-starter organisms, and provides lipases and proteases necessary for flavour development during curing. Further information on LAB and starter cultures can be found in the microbiology section.

After inoculation with the starter culture, the milk is held for 45 to 60 min at 25 to 30° C to ensure the bacteria are active, growing and have developed acidity. This stage is called ripening the milk and is done prior to renneting.

Milk Coagulation

Coagulation is essentially the formation of a gel by destabilizing the casein micelles causing them to aggregate and form a network which partially immobilizes the water and traps the fat globules in the newly formed matrix. This may be accomplished with:

- enzymes
- acid treatment
- heat-acid treatment

Enzymes

Chymosin, or rennet, is most often used for enzyme coagulation.

Curd Treatment

After the milk has gel has been allowed to reach the desired firmness, it is carefully cut into small pieces with knife blades or wires. This shortens the distance and increases the available area for whey to be released. The curd pieces immediately begin to shrink and expel the greenish liquid called whey. This syneresis process is further driven by a cooking stage. The increase in temperature causes the protein matrix to shrink due to increased hydrophobic interactions, and also increases the rate of fermentation of lactose to lactic acid. The increased acidity also contributes to shrinkage of the curd particles. The final moisture content is dependant on the time and temperature of the cook stage. This is

important to monitor carefully because the final moisture content of the curd determines the residual amount of fermentable lactose and thus the final pH of the cheese after curing. When the curds have reached the desired moisture and acidity they are separated from the whey. The whey may be removed from the top or drained by gravity. The curd-whey mixture may also be placed in moulds for draining. Curd handling from this point on is very specific for each cheese variety.

Whey types selection. Procedures

Cheese	Type of Whey	Procedure
Curd brick cheese	Sweet	¦ Milk pasteurization at 62 °C 30 min ¦ Add mesophyllic cheese starter and 2 ml of rennet per 10 kg of milk. ¦ Raise the temperature to 36 °C ¦ Add water at 36 °C ¦ Pour the curd and remaining wash water into the hoops. ¦ Collect whey
European style cottage cheese	Sweet	¦ Skim cream as possible from fresh milk. ¦ Pasteurize skim milk at 62 °C for 30 minutes ¦ Add low temperature cheese starter 0.5 kg/10 kg of milk. ¦ Stir gently and heat slowly to 52 °C. ¦ Drain most of the whey
Cheddar cheese	Sweet	¦ Warm the milk to 32 °C ¦ Add mesophilic starter culture ¦ After a prescribed time slowly raise (45 min). ¦ Cook at 39 °C for another 45 min. ¦ Drain the whey by pouring through a cheesecloth
Latin American white cheese)	Sour	¦ Heat milk to 80 °C for 20 minutes. ¦ Add 1/3 diluted vinegar (175 ml/5 kg of milk) ¦ Separate the curd by filtering through a cloth bag until free whey is removed.

Whey Fermentation

Whey may be demineralised, ultrafiltered, concentrated and dried to recover it most valuable constituents, e.g. lactose and proteins. In all case the large capital investments required and the low market prices of the products make whey upgrading just about enough to cover operation expenses. Among a number of upgrading options proposed, it is worth quoting the utilisation of whey as a substrate for the production of yeast, alcohol, non-alcoholic beverages etc. In particular, *Kluyveromyces fragilis* cultivation has been recommended.

K. fragilis utilise lactose selectively, and as soon as this carbon source is exhausted, it starts to metabolise other whey components as ethanol, esters, glycerol etc.

For the study of whey fermentation two kinds of kinetic models has to be analysed.

1. Yeast biomass propagation for fodder protein production, and
2. Ethanol fermentation for fuel purposes

In the first case, growth pattern can be monitored through COD analysis, as has been done previously in distillery slop fermentation with excellent results (Otero *et al* 2003, Martinez *et al* 2004), and dry matter estimation both as a function of time for yield, batch productivity and specific growth rate (μ). Propagation trials will be done with and without the addition of a Microbial Growth Promoter QZ-350 (Quimizuk, Havana, Cuba). Preliminary techno-economical analysis will be carried out as well.

For ethanol trials, experiments at controlled temperature and nutrient salt addition will be conducted for the calculation of yield and efficiency parameters.

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Martínez, J.A.; Almazán, O.A.; Saura, G.; Otero, M.A. (2004) Production of fodder yeast from stillage in Cuba: an environmental approach *Zuckerindustrie* 129 (2):92-95

2. BATCH PROPAGATION OF *Kluyveromyces* sp YEAST in WHEY

Responsible: Miguel A. Otero-Rambla and Gustavo Saura Laria

Instituto Cubano de Investigaciones de los Derivados de la Caña de Azúcar (ICIDCA)

ABSTRACT

Kluyveromyces fragilis L-1930 was grown on reconstituted whey powder at a lactose concentration of 20 g/L supplemented with inorganic salts as sources of nitrogen and phosphate. The effect of pH in the range of 4.5-5.5 turned out to be non significant respect to specific growth rate (μmax) or biomass-substrate yield. Biomass composition showed values similar to those obtained from different substrates

Key words: *cheese whey, Kluyveromyces fragilis, single cell protein, wastewaters treatment*

INTRODUCTION

Whey is the aqueous fraction of milk generated as a by-product of cheese manufacturing which is produced in large amounts, about 9 L per kg of cheese produced. The main sugar in cheese whey is lactose, at a concentration of about 3-8% (Speer, 1998). Other components are protein, salts and vitamins that are present in minor amounts. The low concentration of these components makes their recovery non atractive from the economical point of view.

Whey is, on the other hand one of the most polluting industrial wastes with a BOD of about 30 kg/m^3, thus if disposed directly to the environment serious contamination problems would be faced (Cristiani-Urbina et al. 2000). As a solution, bioconversion of whey into

SCP or ethanol has been performed in several countries (Mawson, 1994; Gonzales Siso, 1996; Grba et al 2002; Zafar et al. 2005). However, the main problem is that whey sources are different in size and on the contrary of distillery slops are not concentrated in small areas.

The use of whey for the production of yeast biomass has the advantages that it is a simple treatment process, and the final discharge of the whey is facilitated since the pollutant load is significantly reduced and the whey lactose is converted into yeast biomass. The SCP could be produced from whey using yeasts such as *Kluyveromyces, Candida* and *Trichosporon,* as they are naturally able to metabolize lactose, it has been observed that in aerated cultures of *Kluyveromyces fragilis*, *K. lactis* a change in cellular metabolism from oxidative to a mixed oxidative-fermentative state can occurred. This change causes the production of by-metabolic products such as alcohol, aldehydes, esters, etc., which reduce the yields of biomass on whey (Cristiani-Urbina et al. 1997; Ustáriz et al. 2007)

The *Kluyveromyces* species have been most widely studied for SCP production from whey (Moresi et al. 1989; Carlotti et al. 1991; Ben-Hassan et al. 1994; Grba et al. 2002; Cristiani-Urbina et al. 2000). In present paper the ability of a strain of *Kluyveromyces fragilis* for consumption of lactose and yeast biomass production was studied.

MATERIALS AND METHODS

Microorganism. Kluyveromyces fragilis L-1930 was grown batchwise on reconstituted cheese whey as the sole source of carbon and energy to get 20 g/L of lactose in propagation medium supplemented with 0.78 mg/mL diammonium phosphate and 3.75 mg/mL diamonium sulfate as phosphorus and nitrogen sources respectively. Batch at bench scale were conducted at 32 °C, pH 4.5-5.5 and oxygen transfer rate of 80 mM of oxygen per liter per hour in a set of 3 fully instrumented MD5 Marubishi fermentors. Biomass produced in different experiments was centrifuged, washed twice with distilled water and stored at 4 °C until analysis.

Media composition. The medium used was made up with 20 g L^{-1} of reconstituted sweet whey powder, obtained from a local dairy industry. Table 1 shows the composition of whey powder.

Chemical analysis COD was estimated by reflux of an oxidation mixture of sample in presence of potassium dichromate and sulphuric acid (Conde et al 1978). Ashes were determined by sample incineration at 450°C and expressed as carbonates. Nitrogen was

estimated according to Kjeldahl (Anon 1983) in a 1030 Kjeltec Auto System (Tecator AB, Höganäs, Sweden). Phosphorus was quantitated by ammonium vanadate-molibdate methods and read at 415nm. Lipids were extracted in a Soxhlet apparatus with ethyl ether after ultrasonic disruption of yeast cell suspensions and determined by dry weight in a vacuum oven at 60 °C overnight

RESULTS AND DISCUSION

Table 1 shows the average composition of whey used in all trials. Analysis were done by triplicate and non significant variation were observed between batches.

Table 1 Whey powder composition

Component	%
Moisture	3 ± 0.21
Lactose	71 ± 1.12
Soluble proteins	11.1 ± 0.97
Fat	0.7 ± 0.03
Ash	7.2 ± 0.99

K. fragilis uses non protein nitrogen in the whey for growth as has been previously reported (Vanauvat and Kinsella, 1975a, 1975b), but not the heat precipitable whey protein. Thus, the cellular protein of the yeasts apparently is formed from the non protein nitrogen utilized by the yeasts during growth in whey. This provides a means for recovering non protein nitrogen from the cheese whey in a form that can be utilized for a feed supplement or as a food ingredient. However, in order to sustain yeast growth inorganic salts as ammonium sulphate and phosphate should be added to propagation broth as it is normal when other wastewaters treated by biological means (Otero et al 2007, Otero et al 2008).

Fig 1 shows the kinetic growth pattern of *K. fragilis* grown on whey. For industrial implementation, propagation broth has to be supplemented with inorganic salts as sources of phosphorus and nitrogen, to achieve high lactose bioconversion and concomitantly COD removal. Logarithm of biomass values were plotted vs time and a $\mu mx = 0.334\ h^{-1}$ was obtained. Other experiments were conducted at different pH values, results are offered in Table 2.

Fig 1 clearly demonstrated that growth had no significant differences in pH range from 4.5 to 5.5. It is likely to suppose that slightly above and below of that range, changes, if any, will be small respect to biomass concentration and other kinetic parameters.

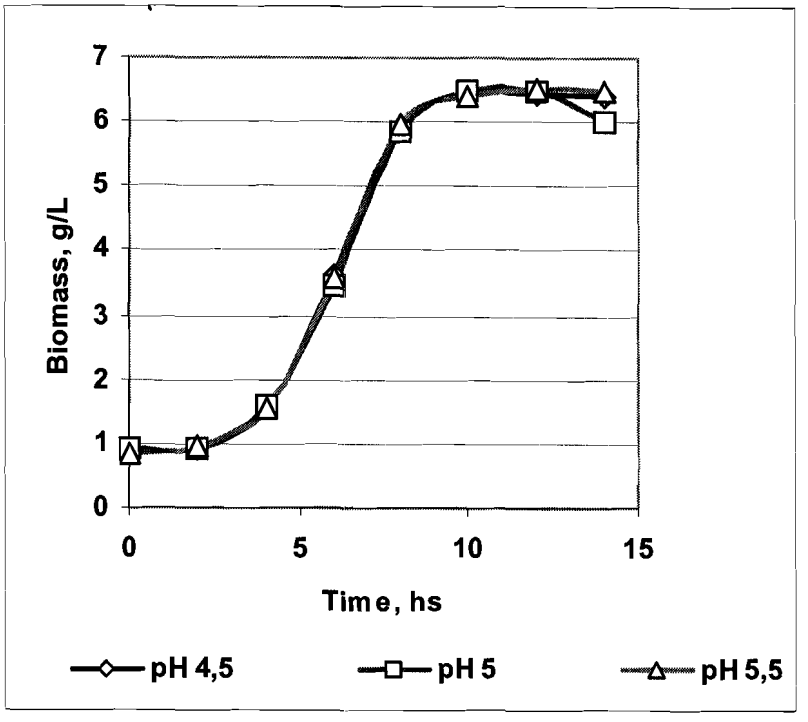

Fig 1 Growth curve of *Kluyveromyces fragilis* on lactose as carbon and energy source. Other growth conditions: pH 4.5-5.5, T 32°C, OTR 80 mM O2/L-h

Table 2 offers the graphic calculation of maximum specific growth rate (μmax), straight line equation for its estimation and correlation coefficients for all pH tested.

Table 2 Graphical calculation of μmax OF *Kluyveromyces fragilis* L-1930 grown on whey as sole source of carbon and energy. Effect of pH. Other conditions were: T = 32 °C, OTR = 80 mM O2/L-h

pH	Line equation	Correlation coefficient	$Y_{x/s}$	μmax, h^{-1}
4.5	y = 0.338X- 0.834	0.9895	0.339	0.334
5.0	y = 0.338X- 0.864	0.9899	0.341	0.338
5.5	y = 0.325X- 0.784	0.9908	0.342	0.325

Values for μmax are lower than those expected for this strain; however, it is possible that even when whey is rich in metabolizable sugars, some co-factors as minerals, vitamins etc., were not in the required amounts for a full display of strain potentialities. Further experiences would be needed with the addition of small amounts of growth enhancers, molasses or any other substrate able to contribute to the enrichment of whey.

Yields respect to sugars fed also show no differences, but as growth rates did, exhibits values below to those expected, probably for the same reason as above.

The biomass obtained from whey was analyzed for its centesimal composition, results are shown in Table 3.

Table 3 Composition of yeast biomass from whey*

Component, percent of dry weight	**Whey yeast**
Kjeldahl protein	45.12 ± 1.15
Nucleic acids	6.25 ± 0.92
Phosphorus (as P_2O_5)	2.87 ± 0.35
Ash	6.52 ± 1.13
Lipids	1.37 ±

* pool of biomass propagated at different pHs

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3. LACTOSE HYDROLYSIS BY ß-GALACTOSIDASE. BEVERAGE DEVELOPMENT FROM HIDROLIZATED (delactosed) WHEY

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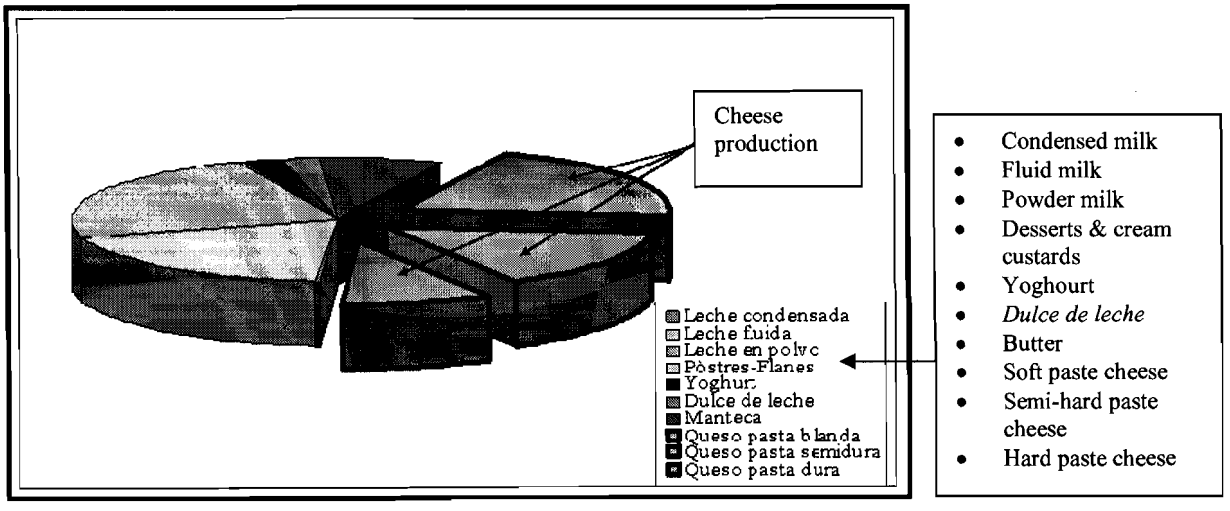
Introduction

Dairy industry is one of the most representatives in Argentina. It is spread mainly in Santa Fe, Cordoba and Buenos Aires provinces which together concentrate 95% of production and in a lesser scale in Entre Ríos and La Pampa provinces (SAGPyA, 2003; CIL, 2003; Cuellas, 2008).

In Argentina, are currently registered about 14.000 *tambos*, which devote around 50% of their production to cheese industry (Figure 1). This growth is the result of a concentration and especialization process that led to a production increase from an average of 200 L/day per *tambo*, up to almost 1.700 L/day at present (SAGPyA, 2003; CIL, 2003; Cuellas, 2008).

Figure 1: Percentage distribution of dairy products in Argentina (SAGPyA 2003)



Cheese comprises today the main destination of milk in Argentina; such an expansion produces great amounts of cheese whey, the sole remnant product in cheese manufacturing process. This effluent is poorly used and it is disposed almost totally in rivers and ponds provoking a huge increase in pollution levels in neighbour ecosystems (Cuellas, 2005).

There are two types of whey that depends on the coagulation system applied in it elaboration: sweet whey, obtained through clog aggregation, with pH 6,5, and sour whey obtained by means of lactic fermentation with pH 5,0. Table 1 shows the Basic composition of the two types of whey (Inda Cuningham 2000; Mammarella, 2001; Cuellas, 2005; Cuellas 2008).

On the other hand, this by-product is at the same time an important source of nutrients, since it exhibits a fairly good profile of minerals, where stand out potassium, calcium, phosphorus and magnesium, high biological value proteins and represents an important source of carbohydrates with low fat and calories content, as well as B complex vitamins (Table 2 and 3) (Inda Cuningham, 2000; Miranda 2007; Cuellas, 2008).

Table 1: Composition of sweet and sour whey

Compound	Sweet whey	Sour whey
Water	93-94 %	94-95 %
Dry extract	6-7 %	5-6 %
Lactose	4,5-5,0 %	3,8-4,2 %
Lactic acid	traces	0,8 %
Protein	0,8-1,0 %	0,8-1,0 %
Citric acid	0,1 %	0,1 %
Ash	0,5-0,7 %	0,5-0,7 %

Table 2: Mineral content in cheese whey

Mineral	Amount, mg/100 g
Calcium	47
Iron	0.06
Magnesium	8
Phosphorus	46
Potassium	161
Sodium	54
Zinc	0.13

Table 3: Vitamin content in cheese whey

Vitamin	Amount per 100 g
Ascorbic acid, mg	47
Thiamine, mg	0.10
Riboflavin, mg	0.036
Niacin, mg	0.074
Panthotenic acid, mg	0.383
Pyridoxine, mg	0.031
Folic acid, μg	1
Vitamin B12, μg	0.027
Vitamin A, IU	16

In Latin America this effluent is scarcely used in animal nutrition, mainly for pigs and bovines. However, in USA and Europe markets a variety of whey-based products as beverages, pharmaceuticals, powder proteins and cheese, among others, began to appear taking advantage of it properties and ameliorating the environmental problems associated to it disposal (Miranda 2007).

The use of cheese whey is limited by transportation costs and its perishability, which makes almost impossible its storage without previous treatment for long periods (Zumbado, 2006; Zalazar 1994). At national scale the milk factories that collect cheese whey are scarce, even less are those that count with the hygienic and sanitary conditions proper for its handling. Therefore, with the implementation of appropriate conditions for its collection and utilization in small or medium size enterprises, a promising future arises for covering nutritional programs and to develop new milk-derived products of commercial interest. In such a way a better use of renewable resources, minimizing at the same time the negative environmental impact will come across.

Thus, the necessity to apply the scientific and technologic knowledge to solve specific industrial problems achieves a paramount importance. The growth of national cheese industry requires innovative alternatives aimed to the improvement of it competitiveness and that lead to the design of new products from the main polluting waste of this sector.

Pre-feasibility study for the elaboration of products from cheese whey

The purpose of present study is to analyze and provide concepts to help cheese-making entrepreneurs in the developing of economical and consumer-appealing products from whey.

Table 4: Analysis of different products from cheese whey

Product type	*Use level*	*Time and production cost*	*Pollution reduction*	*Product acceptability*
Ricotta	Low-medium	Medium	Low	Medium
Protein concentrate	Low-medium	High	Low	High
Mysost-type cheese	High	Medium	High	Low
Isotonic beverages	**High**	**Low**	**High**	**High**

The characteristics and composition of this effluent allow the design of an ample range of options for the development of food products. The criterion of choice for the elaboration of some of them must be adequate to the needs and possibilities of every industrial facility, as well as, to take into account different basic aspects, as processing cost, production time and marketing breakthrough.

Description of analyzed products

- *Ricotta*: is a product obtained by the heat precipitation of proteins in acid medium (Inda Cuningham, 2000).
- *Protein concentrates*: whey protein concentrates (WPC) are obtained by ultrafiltration techniques. They can contain from 15 to 85% of proteins (Chacón, 2006; CPML-N, 2004).
- *Mysost-type cheese*: are commercial products of Scandinavian origin. They have the advantage that all whey solids are used. Production technology is basically a solid concentration process, similar to that applied in *dulce de leche* production (Inda Cuningham, 2000; CPML-N, 2004).

- *Beverages of milky-formulations*: those are low-cost beverages. They can be pasteurized, flavoured, fortified or delactosed.

a- Level of utilization of cheese whey as raw material for different products.

As it was mentioned above, cheese whey is a scarcely used source of nutrients. It contains a little bit more than 25 % of all proteins present in milk, close to 8 % of fats and about 95 % of lactose. The liquid beverage and Mysot-type cheese take advantage of all components of whey. However, other products as ricotta and WPC, even when they concentrate whey proteins, they generate permeates that contains an important amount of original effluent components.

b- Time and production costs

Time and production costs are basic aspects for product choice. Process for the elaboration of beverages, ricotta mysot-type cheese does not require great investment and takes use the conventional equipment of dairy industry. On the contrary, the obtainment of WPC requires higher investment, since equipment for ultra- and nano-filtration is expensive and most of the time exceeds investment possibilities for small and medium size cheese enterprises.

c- Reduction of environmental pollution

Due to the high polluting power of these effluents, the reduction of wastes and product recycling is a critical point for process choice. Ricotta and WPC still generate liquid wastes that keep a high pollution potential, close to that exhibited by original whey. Therefore it is important yet to look for a upgrading use of them.

d- Product acceptability

The above analyzed products aim at different market segments. Due to their nutritional and functional properties, WPC are widely used in pharmaceutical and food industries. Ricotta can be marketed as such or be used for partial replacement of some spread cheeses. Beverages exhibit a high acceptability in sportsmen, pregnant women and children.

However, the insertion of Mysot-type cheese is less likely since Scandinavian formulations have to be adapted to common liking of Latin American markets (Inda Cuningham 2000; CPML-N, 2004).

According to the characteristics of alternatives in Table 4, the most accepted product seems to be whey-based beverages since offer the best advantages and higher chance to match consumer preferences. Similar products as isotonic and energizing beverages agree with current trends for the consumption of natural and functional foods which constitute an expansion market. In addition the elaboration of beverages utilizes efficiently all resources, employ a simple technology easy to implement and reduce almost totally the environmental impact of cheese whey.

Characteristics and trends of product market

From whey as the main raw material several beverage types can be prepared. Among them, those called isotonic beverages exhibit the higher market demand because the beneficial effects they produce when consumed, as well as, their nutritional characteristics. Isotonic beverages are denominated that way because they have sugar and mineral contents that do not modify blood osmolarity.

Isotonic beverages are formulated to mitigate one's thirst, to restore fluids and electrolytes lost during physical exercises, and to provide energy to improve nutritional condition of consumers. Its water contribution prevents dehydration and the carbohydrate concentration from 5% to 8% is quite effective to reduce degradation of glycogen storage, to keep stable blood glucose levels and to speed up water assimilation (Melgarejo, 2004).

Some years ago, the consumption of that kind of products was exclusive of a reduced and selected minority, however, the tendency of standard population to a healthy life and the concomitant consumption of beneficial food, have been exponentially increased. A 90% increase in sales in the latest years is an evidence of this assertion.

Supermarkets show an ample variety of products that include soy-based beverages, energizing soft drinks, powder-like products to prepare vitamin and iron fortified supplements for sportsman, pregnant women, children, etc. Thus, nutritive beverages have extend a market segment by self-adaptation to diet requirements of many people for keep themselves healthy and carry on an active life (Suban, 2006).

The expansion of natural products and the continuous launching of new products, makes a market profile quite dynamic and with higher possibilities. In Argentina, isotonic

beverages have their best opportunities in infants demand, sportsmen and pregnant women, offering a soft drink with a pleasant flavour that can be consumed cold or at ambient temperature. At international scale, there are many whey-based products (generally dehydrated) aimed to this consumers segment, or as dietetic supplement, so it seems to be possible to open a new market line of nutritional products quite attractive to those consumers.

Importance of lactose hydrolysis in product

Lactose is the sole free sugar present in milk. It has a simple chemical structure, is less sweet and soluble than sucrose and in some cases cannot be absorbed by human gastrointestinal track. Lactose is a very scarce sugar in nature; therefore milk is for human beings the unique source of galactose –a lactose constituent-, an energetic monosaccharide constituent of glycolipids and glycoproteins (Alais, 1971).

The split of lactose in glucose and galactose has special importance for human nutrition, given the high proportion of lactose intolerance in some population segments (75 % of black people, 90 % of Asian and American original population, less than 20 % of Caucasoid people from north-eastern Europe and a great part of VIH infected) (Barnes, 1994).

In addition, lactose is a slightly soluble sugar (its solubility is tenfold of that shown by sucrose) and tend to crystallize during storage and thus its utilization as sweetener is not convenient (Hobman, 1984).

When hydrolyzes, lactose releases glucose and galactose, which their combined sweetening power is about 80 % of sucrose. Such hydrolyzate is 3-4 times more soluble than lactose and besides, monosaccharides are absorbed easily by digest mucous membrane (Zadow, 1984).

There are different techniques for lactose hydrolysis and can be carried out by strong acids, ion-exchange resins and by enzymes. Nowadays, there is a boom in the use of enzymes in industry, due to its efficacy, specific action, working conditions and high biodegradability. β-galactosidase or lactase, is the enzyme responsible of lactose splitting, and its action does not affect other components present in milk (Wingrad and co-workers, 1980; Mammarella, 2001; Cuellas, 2005).

β-galactosidase enzyme (E.C. 3.2.1.23), is widely spread in food industry and catalyzes the hydrolysis of lactose yielding an equimolar mixture of glucose and galactose. This enzyme

can be obtained from different sources, nevertheless, its application in food industry is only carried out by the culture of some microorganisms Generally Regarded As Safe (GRAS) by international organisms, among them yeast species as *Kluyveromyces lactis* and *K. fragilis*, as well as, fungi and *A. oryzae* (Office of Pre-market Approval, 1998). In general terms, fungal enzymes are used for lactose hydrolysis in acid whey and yeast ones for milk and neutral whey.

Therefore, sugars obtained from lactose hydrolysis present very interesting properties from technological and nutritional points of views: higher solubility and sweetness, and easy assimilation. This change in properties allows the use of glucose-galactose syrups in a number of applications.

Taking into account above mentioned aspects, the enzyme-catalyzed hydrolysis technique was chosen for the formulation of the new beverage. The obtained product is in good agreement with current trend in dairy market, which pursues the offering of products that keep their whole nutritional properties, but with low lactose content. This product, commonly known as *delactosed* or *hisdrolizados* (Spanish), present nutritional and technological advantages respect to original product enhancing marketing breakthrough possibilities.

The enzyme:

Among present Project objectives, there is the study of preparation and utilization of lactase for its application in sweet whey hydrolysis. With that purpose the most convenient enzymes are those coming from milk natural microbial flora, as it is the case of *Kluyveromyces spp.*, which their optimal conditions for this reaction are from 35 °C to 40 °C, and pH of 6,6-7,3 (Mahoney and Adamchuk, 1980).

In present work for the reaction of splitting of lactose, a comercial preparation of β-galactosidasa (E.C. 3.2.1.23) from *Kluyveromyces fragilis* (Lactozym 3000 L, Novo Nordisk A/S, Denmark). The characteristics of Lactozym 3000 L, are shown in Table 5.

The kinetic model that describes enzyme behavior is Michaelis–Menten model with competitive inhibition by reaction product (Roberts, 1977; Mahoney y Whitaker, 1978).

Reaction scheme is represented in Figure 2

Table 5. Some properties of commercial preparation of ß-galactosidades Lactozym 3000-L

Property	Lactozym 3000L
Aspect	Liquid
Density	1.20 mg/mL
Activity	3000 LAU/ml
Protein concentration	35,09 mg/mL

Table 6. Standard conditions for lactase enzyme activity

Substrate	**4,7 % w/v lactose solution in buffer pH 6,86**
pH	6.7
Temperature	37 °C
Buffer system	Dairy buffer (0,025M KH_2PO_4, 0,025 M Na_2HPO_4 and 1,0 mM $MgSO_4$).

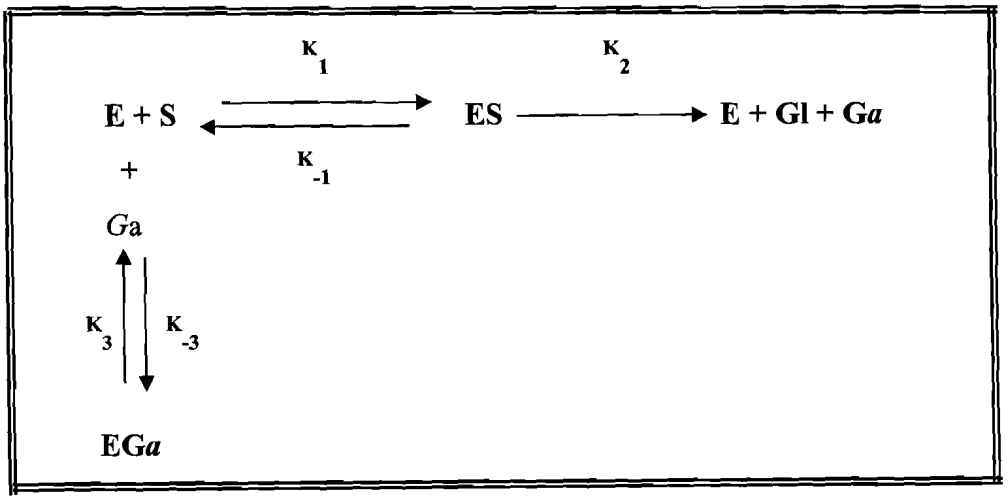


Figure 2 Scheme for enzyme-catalized hydrolysis reaction.

Where E is β-galactosidase enzyme, S is lactose, ES is enzyme-substrate complex (β-galactosidase-lactose), Gl is glucose, G*a* is galactose and G*a* is the enzyme-product complex (β-galactosidase-galactose). The rate of substrate consumption can be expressed through:

$$\frac{d[S]}{dt} = K_1[S][E] - K_{-1}[ES]$$

Where [E] is the concentration of free enzyme, [S] is lactose concentration and [ES] is the concentration of enzyme-substrate complex.

The essays for enzyme activity for the obtainment of kinetic parameters were carried out by monitoring of hydrolysis reactions for lactose solutions and cheese whey. The method of initial rate that indicates that the amount of substrate per time unit is proportional to the amount of enzyme in reaction mixture was used in all experiments. To do so, it has to be guaranteed that the amount of substrate has to be in excess respect to enzyme amounts and that the hydrolysis rate is short enough. These essay conditions prevent enzyme denaturation by one side whereas by the other avoid the decrease of reaction rate by the reduction of substrate concentration due as hydrolysis proceeds.

Hydrolysis degree was determined measuring the amounts of glucose released by enzyme doses, using a reaction kit Wiener Laboratories (Rosario, Argentina) for enzyme determination of *glycaemia*, reading the absorbance at 505 nm in a Milton Roy Spectronic Genesys 5 Spectrophotometer against a glucose standard.

Background

Method fundamentals

Colorimetric reaction follows the scheme shown below:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2$$

$$2\,H_2O_2 + \text{4-AF} + \text{Phenol} \xrightarrow{\text{POD}} \text{4 (p-benzoquinone-mono-imine)} + 4H_2O$$

Where, GOD/POD: is a solution of glucose oxidase (1000 U/ml) and peroxidase (120 U/ml), Reagent 4-AF: corresponds to a solution of 4-amino-pheenazone 25 mmol/l in buffer TRIS 0,92 mol/l. Knowing the amount of enzyme in reaction, specific activity is calculated through the equation

$$Act = \frac{[Gl]V\,10^6}{M\,t\,p^{enz}} \; x\,100$$

where: Act is the specific activity of enzyme defined as the number of μmol of glucose released per minute and per g of enzyme; [Gl] is glucose concentration (in g/l) in sample; V is the volume in litres of reaction mixture; M is the molecular weight of glucose (180 g/mol); t is the reaction time (min) and p^{enz} is the amount of enzyme utilized (g). To allow a fast comparison among different conditions, relative activity is calculated, taking into account the activity determined under conditions recommended by supplier, by the relationship.

$$Act_{r\%} = \frac{Act_e}{Act_s} \; x\,100$$

Where: $Act_{r\%}$ is the relative activity of enzyme, expressed as percentage, Act_e is the specific activity under assay conditions and Act_s is specific activity determined under conditions recommended by the supplier.

Determination of kinetic constants for free enzyme in a batch assay.

At first approach for determination of enzyme kinetic constants, experiments were carried out batchwise using pure lactose as substrate at different initial concentrations $[S]^0$. All assays were done by triplicate at 37 °C, pH 6,8 and constant stirring.

To conduct experiments with free enzyme, four conditions were assayed in which 10 μl of commercial enzyme Lactozym 3000L, were put in contact with 5 ml of lactose solution at initial concentrations of: 2,5; 5,0; 7,5 y 10,0%, respectively. Reaction was incubated at 37 °C, with occasional stirring with a Vortex stirrer. Hydrolysis reaction was stopped at different time intervals by addition of NaOH. Assays were also carried out by triplicate.

Kinetic constant calculation

UIT conversión data and applying initial rates method through non linear multiple regression analysis, the kinetic constants were calculated. To do it, toolbox Simulink from Matlab® software was used and program initializing was done with initial estimation of obtained results as a function of substrate.

Evolving of hydrolysis degree of lactose solutions with free and immobilized enzyme

The evolving of hydrolysis degree of lactose at different initial substrate concentrations $[S]^0$, for free enzyme is shown in Table 7. These results that hydrolysis degree increases as lactose concentration decreases.

Table 7 Lactose splitting by free enzyme at different substrate concentrations

Time (min)	$[S]^0$=2,5%	$[S]^0$=5%	$[S]^0$=7,5%	$[S]^0$=10%
0	0	0	0	0
1	0,060	0,058	0,031	0,027
3	0,170	0,114	0,08	0,06
5	0,300	0,182	0,138	0,110
10	0,505	0,368	0,261	0,211
15	0,669	0,463	0,367	0,312
20	0,799	0,603	0,471	0,353
23	0,850	0,664	0,518	0,423
30	0,910	0,753	0,699	0,502
35	nd*	0,811	0,717	0,598
40	nd*	0,859	0,760	0,637
50	nd*	nd*	0,831	0,752
60	nd*	nd*	nd*	0,805

nd* : Not determined, assays were done up to reach a splitting higher than 80 %

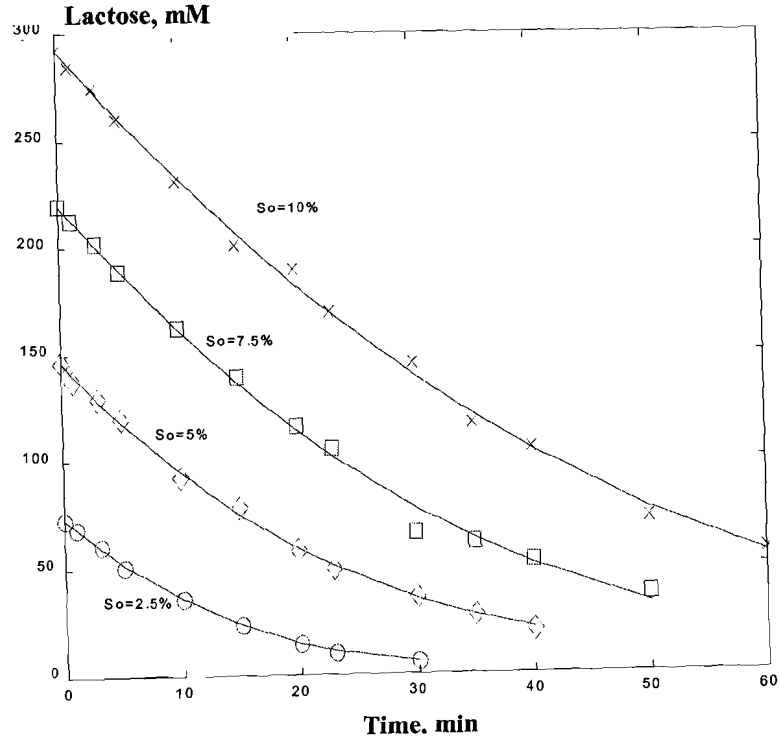


Fig 3 Rate of lactose consumption in batch experiments for free enzyme, at different sustrate concentrations (S_0)

Finally, with experimental data and by applying non linear regression the kinetic constants for enzyme were obtained.

Tabla 8. Kinetic parameters for free and immobilized lactase

Parameters	Free enzyme
Km [mmol l^{-1}]	38.93
K_2 [mmole $lt^{-1}min^{-1}mg^{-1}$]	3.815
K_i [mmol lt^{-1}]	79.207

Beverage development

For product formulation several whey hydrolysates were prepared at different proportions with a range of additives to achieve beverages with good organoleptic properties and high market acceptability. Final decision about formulates were taken according to results given by a trained board.

Materials used in beverage preparation:

- Sweet cheese whey, powder.
- β-galactosidase (Lactozym 3000 l Novo Nordisk)
- Additive mixtures (flavouring and colorant), orange flavour, grapefruit and frutti.
- Sodium benzoate
- Citric acid
- Sucrose

Obtainment of lactose syrups with different hydrolysis degree

In order to assess the appropriate hydrolysis degree for product formulation, cheese whey powder was re-hydrated up to 5% w/v of lactose, as it is found in fresh effluent from cheese making.

Enzymatic hydrolysis of lactose was carried out batchwise with commercial β-galactosidase (Lactozym 3000 l Novo Nordisk) at pH 6.8-7 and 37°C. Reaction was stopped by citric acid addition since enzyme is deactivated at acid pH.

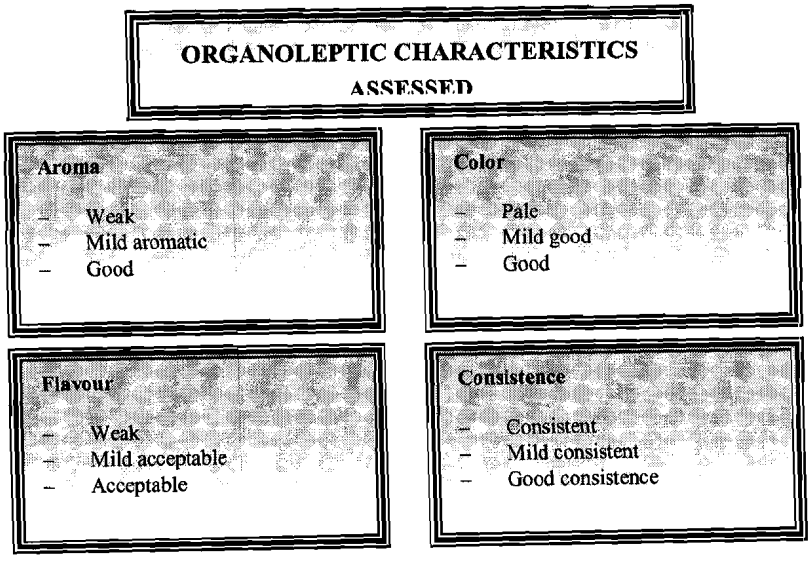
Three experimental lactose syrups were obtained at hydrolysis degrees of 20 %, 50% and 80%. Initial syrup selection was done according to the amount of sucrose needed to confer a sweet flavour. Beverages prepared from whey at different hydrolysis degree (80%, 50% and 20%) will have a constant composition except sucrose that will require low, medium and high additions respectively. Therefore, at higher hydrolysis degree such beverages will offer nutritional and technological advantages, since they will contain higher amounts of monosaccharides and less sucrose and lactose, favouring a faster absorption, less insuline response and lower cost.

Hydrolysis degree in syrups was determined measuring the amount of glucose released by enzyme dosage, through a kit for glycaemia at 505 nm against a Standard.

After the choice of 80% of hydrolysis as the best condition for product elaboration, several beverages were prepared based on this syrup by the addition of different mixtures for different tastes (flavour/color).

To assess sensory board preferences about beverage flavour, the organoleptic characteristics of three fruit flavours were analyzed: orange (formulation I), grapefruit (formulation II) and fruity (formulation III). Fig 4 shows the application designed for sensory assessment.

Fig 4: Organoleptic characteristics assessed by sensory board



In this procedure the use of a preserving product, specially if commercial chain does not guarantees that temperatures will be kept below 4 °C. Taking into account that citric acid addition provides an acid pH and the flavour chosen is citric, the ideal product is sodium benzoate (it improves the activity at acid pH) and maximum dose is 0.1 %. It have to be

mentioned that the function of a preserving compound is to keep unchanged the quality already existing in the product, never to improve it, so it is essential to carry out the whole product development process by Good manufacturing Practices (GMP).

The formulated beverages was pasteurized and submitted to the following microbiological analysis corresponding to Argentina Food Code:

-Counting of total coliform microorganisms at 30°C - NMP (ICMSF, 2000)

-Counting of fecal coliforms and *E. coli*;

-Counting of mesophilic microorganisms (ICMSF, 1983);

-Counting of *S. aureus* (Met. 975.55 AOAC 1995).

Discussion

Sensory assessment

Figures 5, 6 and 7 show the results obtained by sensory assessment for all flavour tested. This study reveals a clear preference to citric flavours (orange and grapefruit) in isotonic beverages. On the other hand, colour and aroma showed better properties and therefore it was the flavour embodied to final formulation. It is important to notice that all evaluated aspect in Formulation I, a mark of 70% or higher, which qualified the beverage procedure as a viable option.

Fig 5: Results for sensory assessment of orange-flavoured beverage

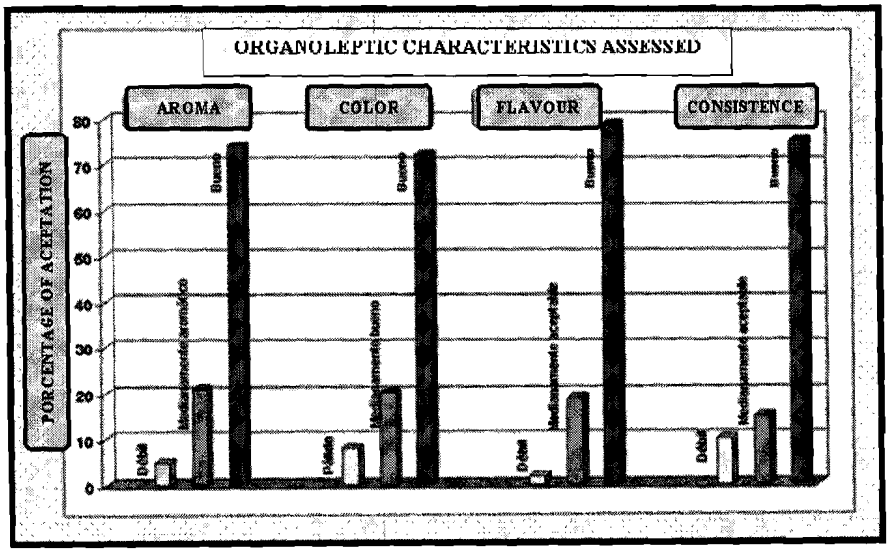


Fig 6: Results for sensory assessment of grapefruit-flavoured beverage

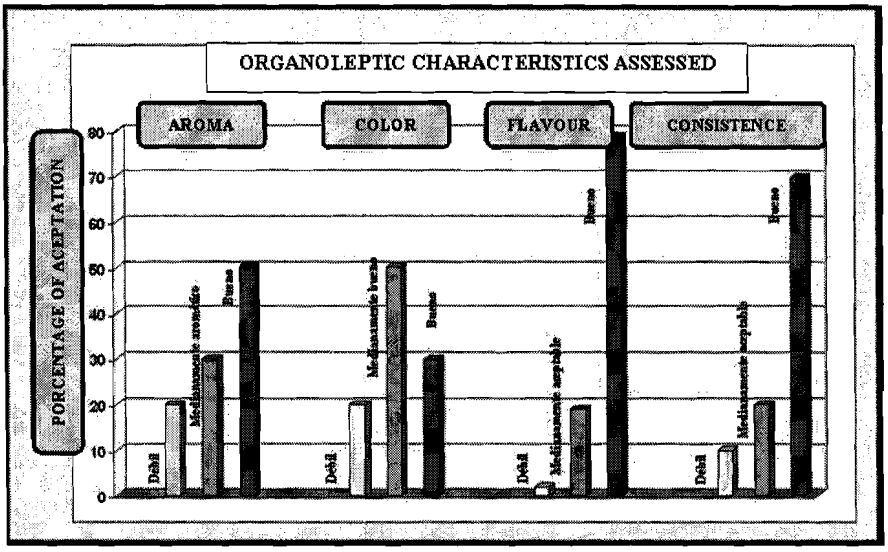
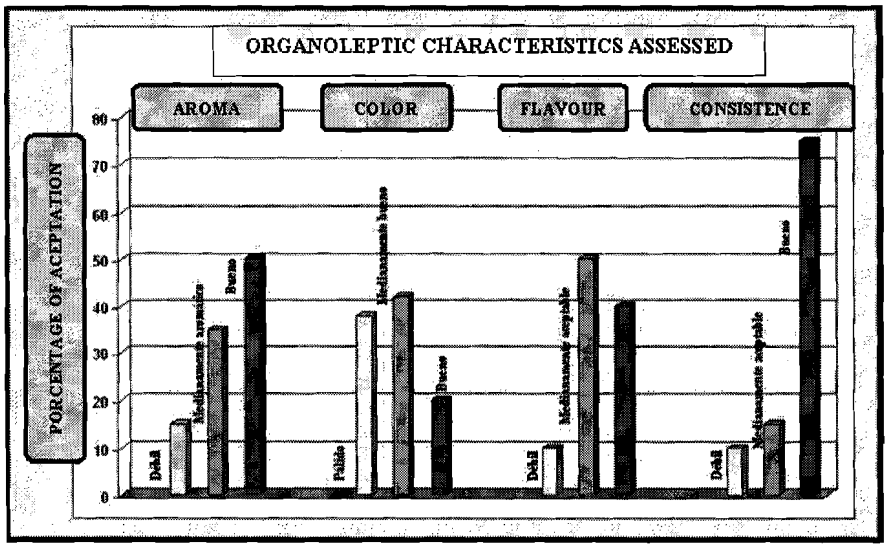


Fig 7: Results for sensory assessment of frutty-flavoured beverage



Description and characteristics of final product

Final product corresponds to a hydrolyzed milky beverage for human intake. By its mineral and carbohydrate contents it classify as an isotonic beverage (Fig 8), contributing with 56 Kcal per 100 ml of product (Fig 9).

Fig 8: Characteristics of final product

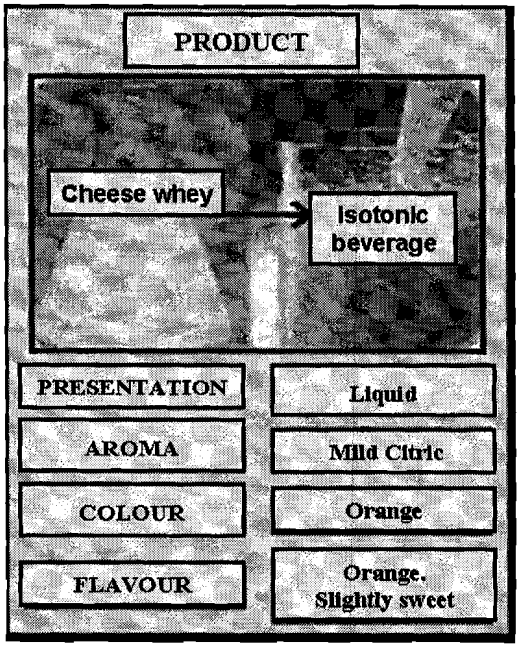


Fig 8 : Nutritional information of final product

NUTRITIONAL INFORMATION		
CHEESE WHEY BASED ISOTONIC BEVERAGE		
	Qty /100 mL final product	% RDA*
Energy value, Kcal	56	2.8
Carbohydrates, g	13	4.33
Protein, g	1	1.33
Total fats, g	Non significant	-----
Saturated fats, g	none	-----
Unsaturated fats, g	none	-----
Sodium, g	54	2.25
Minerals, g	0.5	-----
* Recommended Dietary Allowances based on 2000 Kcal/day diet. Your dietary values could be different according to your energy needs		

Microbiological results

Microbiological analysis according to Argentina Food Codex is detailed below. These results show that the new beverage is apt for human intake

Total coliforms: NMP< 3

Faecal coliforms, *E. coli*, and *S. aureus*: Negative

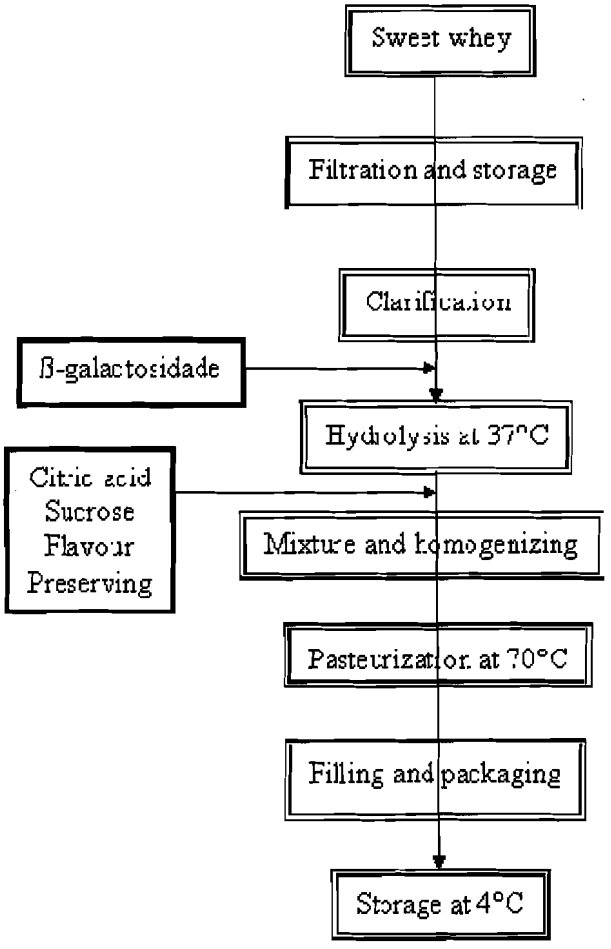
Counting of total mesophilic microorganisms: < 10 CFU/ ml

Process for the preparation of cheese whey-based beverage.

The procedure for the preparation of an isotonic beverage from cheese whey in simple and require equipment typical in dairy industry, thus this process can be easily embodied to small and medium enterprises coupled to production line in cheese making factories.

Fig 9 proposes a flow diagram for beverage production at industrial scale, different to that assayed at lab scale, for the commercial implementation.

Fig 9: Flow diagram for isotonic beverage preparation



Beverages obtained, are pasteurizad and hot packaging is strongly recommended, at the same temperature to that used for pasteurization Ander a controlled microbiologically

conditioned area. Packaging should be in glass or plastic bottles previously sterilized, with screwed hermetic stopper.

Conclusions

From a feasibility study for the elaboration of a whey-based product it is concluded that the preparation of an isotonic beverage is the most appealing option, due to process simplicity, integral utilization of effluent and potential market.

A highly acceptable product by a sensory board was obtained with good organoleptic properties as well as, ample possibilities to be inserted in national market.

Obtained product is aimed basically to sportsmen, children and pregnant women and it is inserted in a market of energizing and isotonic beverages in current expansion.

Elaboration procedure does not present any difficulty and uses common installations and equipment in dairy industry.

The implementation of this technology will be helpful to cheese making entrepreneurs for the reduction of environmental impact concomitant to the indiscriminated disposal of cheese whey. In sucha way, the investment is justified by matching environmental regulation for cheese making industry and the increase of profitability through output diversification.

Taking into account that for the industrial development in our region, it is fundamental the implementation of management systems that bring together both the integral utilization of resources and environmental protection, namely sustainability, it is concluded that the production of that kind of beverages offers the possibility to carry out more efficient processes, through cleaner technologies and with higher profits.

Expected impact:

There are in Latin America a great number of small and medium size cheese making factories that cannot carry on great investment so, the implementation of production lines for whey drying with associated lactose recovery installation and/or whey concentration is out of the reach of such enterprises. The utilization of this waste as raw material for food products will be a feasible and easily implemented solution to improve current process and obtaining new highly upgraded products.

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4. PRODUCTION AND TESTING OF INMUNOESTIMULANTS

IMMUNOLOGICAL ACTIVITY OF SOME POLYSACCHARIDES FROM YEAST CELL WALLS OF Kluyveromyces Fragilis

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Abstract

Polysaccharides from the yeast Kluyveromyces fragilis were extracted with: boiling water (FI), 3 % ammonium oxalate (FII) and 5 % NaOH (FIII). The latest was further fractionated into two different species by neutralization (FIII-1) and ethanol precipitation (FIII-2). From the obtained polysaccharides, three of them exhibited the best behaviour as vaccine adjuvant (FII, FIII-1 y FIII-2) showing a low or none toxicity and high immunological titre in comparison with a commercial batch with controlled immunogenicity. Fractions were structurally analyzed: FI resulted to be a glucomannan with two subfractions with molecular weight of 90 and 6,5 KDa; FII was also a glucomannan with ß(1-3) bonds and two subfractions of 70 and 7 KDa; FIII-1 was a ß-glucan with a molecular weight distribution of 3500, 875 and 65 KDa; FIII-2 resulted the

most homogeneous fraction with a sole peak corresponding to 82 KDa with an overwhelming mannose composition.

Key words: polysaccharides, yeast, Kluyveromyces fragilis, characterization, immunological activity

Introduction

It is known for many decades that macromolecular components of microorganisms as polysaccharides, glycoproteins and nucleic acids exhibit immunostimulants activity in plants, animals and human beings (Goulet; Cochram and Brown, 1960). The most outstanding compounds are homopolysaccharides, which in addition present low or none collateral effects and are specially gifted for the development of new antitumoral, antiviral drugs for therapeutic treatment (Kogan, 1988; Susuki, 1990; Mizuno 1990; Ma, Mizuno and Ito, 1991; Mizuno, 1992).

First researches on yeast cell walls were produced in the 50s, about non-alkali soluble glucans from baker's yeast (Bell and Northcote 1950; Peat, Whelan and Edwards, 1958) and up to date accumulates a vast bibliography until present day. (Manners and Patterson, 1968; Bacon and Farmer, 1968; DiLuzio and Williams, 1978; Duffus, Levi and Manners, 1982; Williams, 1985, Hromadkova et al, 2003, Freimund et al, 2003; Liu et al, 2008).

Kluyveromyces fragilis yeast does not present pathogenicity and has been deeply studied in nutritional and toxicological aspects L (Klibansky, 1986; Hernández, 1989). Nevertheless, the study of this specie as a source of polysaccharides with biological response activators have no been previously published (Sánchez, Martínez and Fuentes, 1987, 1988).

Present paper reports the isolation, purification and partial characterization of different structural polysaccharides from K. fragilis, as well as, their acute toxicological assessment and immunological activity as adjuvant for a vaccine complex. Some physical-chemical properties of isolated compounds are also
described.

Materials and methods

Microorganism. The strain Kluyveromyces fragilis L-1930 growth on cane molasses as the source of carbon and energy was used for all experiments. The medium was supplemented with inorganic salts diammonium phosphate and sulphate. Temperature was settled on 33°C, OTR in 80 mM of oxygen /L-h and pH 4.5. Yeast biomass was centrifuged at the end of propagation, washed twice with distilled water and stored until use at 4°C.

Extraction, fractionation and purification of polysacharides. The scheme used is shown in Fig 1. Each fraction was used in experiments as lyophylized powder. Acute toxicology It was carried out on OF-1 mice and guinea pigs (Hartley, CENPALAB, Havana, Cuba) in which a sole dilution from each fraction was applied (160 mg/Kg of living weight) according to Cuban Standard NC 2645 of 1982. Six mice and 2 guinea pigs were inoculated per each fraction tested. Animals were evaluated from the micro and macroscopic points of view through an anatomopathological study.

Compared immunogenicity. Mice from the line Balb C were used in experiments, testing three proportions from each fraction mixed with a vaccine complex (positive control) and physiological fluid. Physico-chemical porperties and structural analysis. Sugar composition of polysaccharides was determined through derivatization as alditol-acetate (Laine, Esselman and Sweeley, 1972) in a Sigma 3 GLC provided of FID detector (Perking Elmer, USA). Separation of compunds was done in a 3 m x 2 mm column packed with 3 % Supelcoport 100-200 mesh column. Temperature for injection and detection was 250°C using N2 as carrier (30 mL/min). Experiments were carried out with a temperature program from 200°C to 230°C at 10°C/min. Chemical analysis Total carbohydrate content of samples was determined by phenol-sulfuric method (Dubois and others, 1956) in previously hydrolyzed samples. Protein according to Lowry (Lowry and others, 1951). Molecular weight of fractions was determined by gel chromatography in a El peso molecular de las fracciones por cromatografía en gel en una 1.6 cm x 1 m Sepharose CL-4B packed column (Pharmacia Biotechnology Int., Sweden) calibrated with dextran of defined molecular weight (Pharmacia Biotechnology Int., Sweden). Ion exchange chromatography in a K16/20 column (Pharmacia Biotechnology Int., Sweden) on DEAE-Cellulose (Fluka-Chemie, Buchs, Suiza). IR spectrum were carried out in KBr tablets in a Perkin Elmer Mod 457 IR spectrophotometer.

Results and Discusion

Fraccionation and biological activity

Moist yeast biomass was washed with 85 % ethanol at 80 °C to remove low molecular weight components. This fraction was neglected. FI, FII and FIII fractions were extracted according to the method showed in Fig 1. Hydrosoluble fraction was further subfractionated and resulting subfractions purified through DEAE Cellulose. Only original fraction was assayed in its biological activity. This fraction yielded three components: the first one eluted before the salt gradient and corresponds to neutral polyssaccharides. Conformed 15% of total sample amount. The other two componentseluted under gradient action and only the first of them showed good resolution (70 % of total applied in column). This result differ from that reported for Basidiomycetes (Ma, Mizuno and Ito, 1991; Mizuno and others, 1992).

Water insoluble polysaccharides (FII) were precipitated with ethanol, dialyzed and lyophylized without further fractionation. Alkaly–soluble fraction (FIII), was neutralized yielding subfraction FIII-1. The addition of ethanol 3:1 to remaining supernatant yielded subfractión FIII-2.

Samples was submitted for acute toxicity assays. Table 1 shows the obtained results.

TABLE 1 Acute toxicology of isolated fractions

Fraction.	Num. Animals..	Initial weight, g	Final weight, g	Increm., g	Evaluation
Mice OF-1					
F-I	6	126.0	169.0	43.0	Satisf.
F-II	6	122.0	136.0	14.0	Satisf.
F-III-1	6	124.0	155.0	31.0	Satisf.
F-III-2	6	119.0	158.0	39.0	Satisf.
Guinea pigs					
F-I	2	334.0	365.0	31.0	Satisf.
F-II	2	322.0	346.0	24.0	Satisf.
F-III-1	2	311.0	356.0	46.0	Satisf.
F-III-2	2	312.0	364.0	52.0	Satisf.

All fractions resulted to be non toxic. Anatomopathological observations showed the presence of characteristic injuries due to the deposit of polysaccharide. Immuniogenicity test are offered in Table 2.

Alkali soluble fractions (FIII-1 y FIII-2) showed high activity specially in those intermediate doses. These results corroborate the immunoadjuvant effect of (1,3) ß-D glucans (Sánchez, Martínez & Fuentes, 1988).

TABLE 2 Inmunogenicity of polysaccharides from cell wall of Kluyveromyces fragilis L-1930

Fraction	**Concentration**	**IgG post inoculation**	
		24 days	**35 days**
F-I	Low	4798	5364
	Medium	4028	3859
	High	3985	4664
F-II	Low	4821	2400
	Medium	4055	3982
	High	2214	3249
F-III-1	Low	9409	3249
	Medium	11374	5248
	High	8321	6128
F-III-2	Low	9811	11533
	Medium	10852	13098
	Alta	15629	12629
Batch 1	Vaccine	6721	3870
Batche 2	Vaccine	2422	6744
Control	-----	0	0

Physico-chemical properties of polysaccharides Table 3 shows some properties of studied fractions. High yields were observed for FIII-1, better than those reported for S. cerevisiae (Williams & DiLuzio 1980) and H. serotina (Ma, Mizuno and Ito, 1991) and a significant level of impurities in all crude samples except FIII-2 which exhibits a carbohydrate content near to 95 %. Periodate oxidation showed the presence of other bonds different than ß(1,3).

TABLE 3 Some properties of yeast polisaccharides from *Kluyveromyces fragilis* L-1930

Fraction	Yield, %	Carbohidr., %	IO_4^- mol	Component	
				Glucan	Mann
F-I	3.51	50.2	0.40	1.3	25.1
F-II	2.46	48.2	0.93	1.4	18.3
F-III-1	11.46	60.5	1.48	61.4	4.7
F-III-2	3.30	95.0	1.20	9.8	38.2

In all samples molecular weight distribution was carried out by gel filtration chromatography. Table 4 offers MW ranges of subfractions.

TABLE 4 Molecular weight distribution of polysaccharide fractions of *K. fragilis*

Fraction	MW, kDa
F-I	Peak 1: 90, Peak 2: 6.5
F-II	Peak 1: 70, Peak 2: 7.0
F-III-1	65
F-III-2	82

Hydrosoluble polysaccharides gave two species with different molecular weight. FII sample shows the same proportion than that reported by Mizuno et al (1990) for Basidiomycetes albeit in yeast it show a higher value. FIII was previously separated into two subfractions. Only FIII-1 resulted to be constitued by three species of different sizes. FIII- 2 exhibted a great homogenity with an unique peak at 82 KDa. The composition of these peaks differ from that reported to H. serotina (Ma, Mizuno and Ito, 1991), where three peaks were found for FIII-1 and two for FIII-2. Table 5 offers the most outstanding bands in IR absorption spectrum. The most probable asignation are also pointed out. The small band at 890 cm-1 suggests the structure of ß-glucan in FIII-1 sample.

Water extraction of yeast cell wall gave a fraction extremely heterogeneous that was furtherly fractionated into two fractions of 90 y 6.5 KDa respectively by gel chromatography (Table 4). The studies carried out by ion exchange chromatography yielded three subfractions. Respect the raw fraction a study about the influence temperature and extraction time. Table 6 offers the obtainesd results.

IR spectroscopy did not reveal differences in functional group composition among the different species. However, extraction level are a direct function of both variables.

TABLE 5 Main bands in ir spectrum of studied polysaccharide fractions

Fraction	Band, cm^{-1}	Asignation
F-I	810 and 915	Glucomannan
	880	ß-mannopiranose
	780	ß(1-3) bond
F-II	810 and 915	Glucomannan
	880	ß-mannopiranose
	850	a-polyglucosan
F-III-1	890	ß-glucan
F-III-2	810 and 915	Glucomannan
	850	a-polyglucosan

Water soluble polysaccharides

TABLE 6 Composition and functional groups of watersoluble polysaccharides from *Kluyveromyces fragilis*

T, °C	T, min	Carbohydr, % (w/v)	MS % (w/v)	Protein, %	A	B	C	D	E
10	0.58	1.37	0.09	0.42	+	+	+	+	+
95	0.80	1.62	0.13	0.49	+	+	+	+	+
180	0.83	1.82	0.26	0.45	+	+	+	+	+
10	0.77	1.66	0.25	0.46	+	+	+	+	+
95	0.98	1.80	0.34	0.54	+	+	+	+	+
180	1.11	2.13	0.44	0.52	+	+	+	+	+
10	0.91	1.76	0.51	0.52	+	+	+	+	+
95	1.42	2.49	0.75	0.57	+	+	+	+	+
180	1.69	2.62	0.89	0.63	+	+	+	+	+

A: 810 cm^{-1}; B: 863 cm^{-1}; C: 914 cm^{-1}; D: 1237 cm^{-1}; E: 1650 cm^{-1}

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5. PRODUCTION OF PIGMENTS FROM YEAST

ASTAXHANTINE BY *Phaffia rhodozyma* FROM CHEESE WHEY

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Introduction

Carotenoids are pigments with importance in food industry since they confer an attractive colour to an ample variety of foods. Besides, they are precursors of Vitamin A as well as, exhibit antioxidant properties quite important from medical point of view.

The role of antioxidants is to neutralize free radicals acting as electron donors, preventing in that way the damage that these radicals produce in living cells. Antioxidants improve immune system, give protection against cancer and have a very important role in the prevention of degenerative diseases. A recent discovery has pointed out that carotenoids have anti-carcinogenic activity and can act as cancer prevention agents in those people with lack of pigmentation.

Carotenoids also have chronic-disease prevention properties, thus the demand for such pigments have dramatically increased in the last few years. Carotenoids are widely distributed in plants, animals and microorganism. Mostly they are responsible for the colour they show. Besides, they play important metabolic roles as light storage in photosynthetic organisms, photo protection, antioxidants and membrane fluid regulation.

Chemical properties and carotenoids types

Chemically speaking, carotenoids lie within terpenoids family and are composed by isoprenoid units. Terpenoids are derivatives of acetate through the biosynthetic route for mevalonic acid, a precursor of such molecules. They are C_{40}-compounds although higher and smaller molecules can be found. About 600 different carotenoids are known present day (obtained form plants and microorganisms) and only a few of them have been industrialized, for instances, licopene, an acyclic carotenoids, bicyclic carotenoids, ß-carotene and oxygenated bicyclic carotenoids as xanthophiles like astaxhantine and cantaxhantine. Carotenoids can be produced by chemical synthesis, by fermentation or by extraction from natural sources (plants and crustaceans). Carotenoids from chemical synthesis are poorly accepted by final consumer; therefore, those natural carotenoids – extraction or fermentation- have a higher demand.

Plants, algae and microorganisms synthesized a great diversity of carotenoids.

They are produced by some microorganisms as bacteria *Mycobacterium lacticota* and *Brevibacterium spp.*, microalgae like *Hematoccocus pluviales*, *Neochloris wimmweri* and *Chlamydomonas nivalis*, fungi as *Peniphora spp.* and by yeasts as *Xanthophyllomyces dendrodhous*, formally named *Phaffia rhodozyma*, *Rhodotorula glutinis* and *R. rubra*. Present day the commercial production of carotenoids is carried out through the algae *Hematococcus pluvialis* and *Phaffia rhodozyma* yeast. The production of carotenoids has been studied in *Rhodotorula glutinis*. Carotenoids production by this yeast is reported using as carbon source agro industrial wastes as grape must and corn step liquor. Due the fact that most of these substrates have important amounts of starch, some studies growing *R. glutinis* together to other amylolytic yeasts have been carried out aiming to the improvement carotenoids production. It has been demonstrated as well, the production of carotenoids by *Rhodoturula rubra* GED8 grown on cheese whey in a mixed culture with lactic bacteria.

Chemical properties and uses of astaxhantine

Astaxhantine (3,3-dihydroxi-ß,ß-carotene-4,4'-dione) is a pigment from the group of carotenoids. Its molecular formula is $C_{40}H_{52}O_4$ with a boiling point of 224 °C. It is insoluble in water but dissolves at ambient temperature in some organic solvent as acetone, dichloro-methane, chloroform and dimethyl sulfoxide. This pigment is responsible of the color reddish-orange of several marine organisms, including fish and crustacean, as well as, some fowls as flamingos. Astaxhantine is a pigment authorized by Food and Drug Administration of USA for salmon species (*Salmonidae*). Therefore, astaxhantine is widely used in as feed additive for trout and salmon. Thus fishes get the appropriate pigmentation degree according to consumer preferences.

At the same time, astaxhantine is a potent antioxidant. It is known that can be 1000 times more effective than Vitamin E in this role. Due to its antioxidant power, astaxhantine could be potentially used in pharmaceutical formulations for human beings.

***Phaffia rhodozyma* and astaxhantine production**

This yeast belongs to the Basidiomycetes group and was originally isolated at the beginning of 70's from tree exudates in mountain regions of Japan and Alaska. Its main features are: it forms reddish-orange colonies due to the presence of carotenoids; it

ferments glucose and other sugars including hemicellulose, xylose, starch, maltose, raffinose, glycerine among others. It cannot assimilate lactose, galactose, glucosamide, D-ribose and D-arabinose.

This yeast is aerobic-facultative a presents sexual and asexual reproduction. *P. rhodozyma* is the asexual state of the yeast, whereas it sexual state has been reported as *Xanthophyllomyces dendrorhous*. Lately the existence of several species of *P. rhodozyma* was demonstrated and not all of them show a sexual state or at least it is not known so far.

The effects of light, temperature and carbon source on astaxhantine production in *P. rhodozyma* have been studied. On the other hand, it has been demonstrated that when the yeast is grown under oxidative stress the amount of astaxhantine is significantly increased.

The effect of light in astaxhantine production by different strains of *P. rhodozyma* grown on xylose, yielded higher amounts of astaxhantine in comparison to results obtained in darkness. For instances *P. rhodozyma* ATCC 24228 produced 2.13 mg of astaxhantine/ml in Light and only 1.38 mg in darkness.

It has been reported that environmental conditions regulate astaxhantine biosynthesis and that this pigment protects *P. rhodozyma* strains from reactive species of oxygen. For astaxhantine production some agro-industrial wastes can be used as carbon source (lignocellulosics and hemicellulose hydrolysates as well as by-products from ethanol distillation). Some authors have reported the production of astanxhantine by *P. rhodozyma* using glycerol as carbon source. Other authors have proposed sugarcane juices, by-products from corn maceration and coconut water for astaxhantine production by that yeast. These authors have modified some culture conditions in connection with substrate used and in general terms they reported higher production of astaxhantine when it is carried out on complex substrates than when glucose is used as carbon source. For instances, *P. rhodozyma* grown on coconut water produced 1.85 mg of astaxhantine/g of cells, while when it was cultured on YM medium (yeast extract, malt and 10 g/l of glucose) produced only 1.05 mg of astaxhantine/g of cells. The use of *P. rhodozyma* as additive for animal feeding has been proposed, mainly for fishes. To do so, yeast has to suffer an acid/thermal treatment followed by dehydration.

Biosynthesis and regulation of astaxhantine by P. rhodozyma

Astaxhantine is a tetra-terpen from the family of poly-isoprenoids that are synthetized by two different pathways, through mevalonate and no-mevalonate (o

pyruvate/glyceraldehide, 3-phosphate). Any of these pathways lead to the formation of the first unit of isoprene, isopentyl pyro-phosphate (IPP). From this compound chain elongation takes place by subsequent condensation (head-tail) of terpenoids to form dimethyl-alyl pyro-phoshate (DMAPP) which is the reactive isomer of IPP to form chains of poly-prenyl pyro-phosphate. The size of these chains is variable. Shorter ones give geranyl pyro-phosphate (C10), medium sized chains yields farnesyl pyro-phosphate (C15) and longer ones form geranyl-geranyl pyro-phosphate (C20). Al,of them are precursors of mono, di and tri terpenes, and carotenoids.

By condensation of two residues of geranyl-geranyl-PP, phytoene is produced. These chains undergo desaturation and form other compounds with conjugated double bonds as licopene, ß-carotene and astaxhantine which have 13 conjugated double bonds. Those molecules exhibit intense colour and act as photo receptors. Biosynthetic pathway for astaxhantine in *P. rhodozyma* seems to involve both, synthetic and degradative systems regulated by oxidative stress. The exposition to O_2-reactive species (O_2 singlet) quickly changes energy levels of carotenoids intermediates and different degenerative products are formed as a response to peroxyl radicals. *P. rhodozyma* presents a sophisticated system capable of change very quickly the carotenegenic capacity that depends on ambient conditions and cell physiology. The biosynthesis of astaxhantine by *P. rhodozyma* is induced by oxygen-reactive species.

Experimental development

Phaffia rhodozyma was obtained from Biotechnology Department of Institute for Biomedical Research from National Autonomous University of Mexico. The strain was conserved in assay tubes with screwed stoppers and synthetic medium YM; incubated at 22 °C for 48 hours. For production, the used media were YM and YPG. Cell suspension from a single tube was poured into a invaginated erlenmeyer of 250 ml capacity with 50 ml culture broth YM and YM + 1=% cheese whey (previously hydrolyzed with lactase at pH 4.5; see Table 1). The inoculated culture broth was incubated at 22 °C, 150 min^{-1} for 48 hours in a thermostated shaker.

Cell biomass in exponential phase was poured into an erlenmeyer with fresh medium at 10-fold inoculum ratio. Cell growth was monitored by dry weight determination.

Table 1. Culture media and propagation

Ingredient	Medium	
	YM	YM+ whey
Hydrolyzed cheese whey	---	10%
Glucose	10g/L	20 g/L
Yeast extract	3 g/L	3 g/L
Bactopeptone	5 g/L	10 g/L
Malt extract	3 g/L	---
Distilled water	1 L	1 L

Total xanthophyles were determined by spectrophotometric method. Solvent-resistant quarz cells were used and absorbance measured at λ= 474 nm in a Beckman DU 650 spectrophotometer. Absorbance values were substituted in formula reported for total xanthophyles:

$$\text{Total xanthophyles (mg/kg)} = (A \times D / P \times 236)\ (1000)$$

where: A: absorbance at 474 nm, D: dilution factor, P: simple weight in g, 236: specific absorbtivity for trans lutein, 1000: conversión factor to mg/kg.

Translutein is taken as reference for total xanthophyles. The dilution factor established was identical to that used in solvent systems: 125 mL (100 mL initially + 25 mL from washing)/10 g of shrimp residue (v/w) = 12.5. Astaxhantine concentration was determined reverse phase HPLC under isocratic conditions with mobile phase acetonitrile /chloroform/ methanol/ water/ propionic acid (71:22:4:2:1), at 1 mL/min. Analysis were carried out in simples taken out every 20 hours, from 0 to 120 hours.

RESULTS

Figure 1 shows cell growth and astaxhantine production in YM and YM + Cheese whey (AC) media. Astaxhantine concentration increases in early growth stages up to exponential phase. After this phase, its increase still goes on up to stationary phase since it is a secondary metabolite.

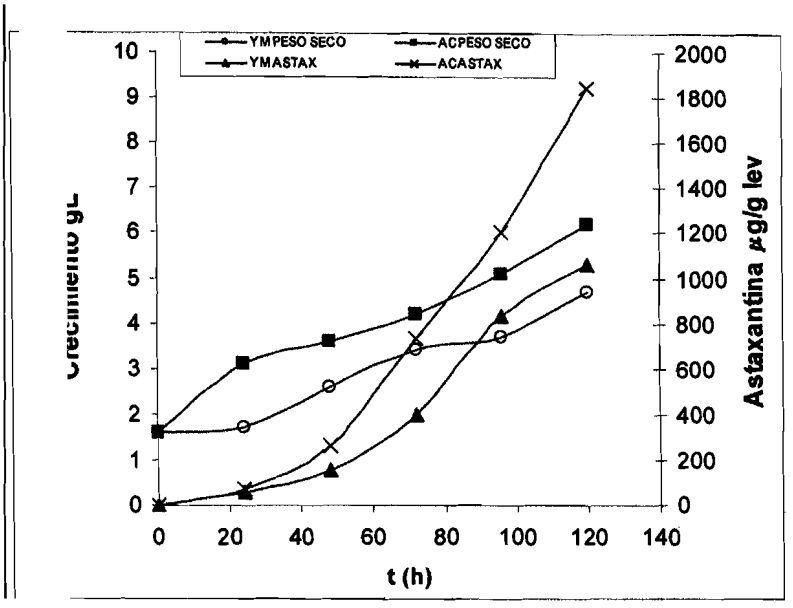

Figure 1 Cell growth and astaxhantine production in YM and YM + cheese whey (AC) media

Astaxhantine production depends on concentration of carbon and nitrogen sources, since values obtained from cheese whey are significantly higher to those reported in technical literature. Therefore, hydrolyzed cheese whey, aiming to make available constituent monosaccharides, could be used as growth medium for *P. rhodozyma* as well as, the production of astaxhantine.

6. PRODUCTION AND TESTING OF EMULSIFIERS

THERMAL AND SURFACE BEHAVIOR OF YEAST PROTEIN FRACTIONS FROM *SACCHAROMYCES CEREVISIAE*

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Abstract

An easy and inexpensive method of fractionation of a yeast homogenate was proposed and it is based on differential centrifugation steps of insoluble components and subsequent precipitations of soluble fractions. In this fractionation, the effect of addition of protease inhibitor (phenylmethylsulfonylfluoride, PMSF) was studied. The procedure, which was performed in mild conditions in order to minimize protein denaturation, allowed the obtention of four fractions that proceed from distinct parts of the yeast cell and with a different composition: Fr I, Fr II, Fr III and Fr IV. Each fraction was chemically characterized and their surface and thermal behavior were also analyzed. Fr I and Fr II, mainly composed by cell wall debris and membrane cell components, respectively, exhibited better surface activity than Fr III and Fr IV, composed by nucleoproteins and citoplasmic proteins. All fractions exhibited an unique DSC endotherm with different T_p and ΔH values. Fr I and Fr II are the fractions with higher entalphy values when they were obtained in presence of protease inhibitor. In absence of PMSF, the structure of Fr I and Fr II were markedly affected reducing their entalphy values and increasing their surface properties.

Introduction

Yeasts, specifically the *Saccharomyces* genus, have historically been recognized for their fermentative capabilities. Traditionally, these unicellular fungi have been used by the food industry principally for their production of ethanol and carbon dioxide which is important to the brewing, wine, distilling and baking industries. At present, yeasts are acquiring increasingy more attention for their other uses (Dzeziak, 1987a). Yeast biomass, as a byproduct of food industry, is a world-spread commodity and contains about 50 % of its dry weight as proteins and other commercially important components, as polysaccharides that could be isolated for the upgrading of yeast production (Dzeziak, 1987b; Otero, Wagner, Vasallo, García, & Añón, 2000; Otero, Wagner, Vasallo, García & Añón, 2002). The limiting factors in the utilization of yeast biomass are its high nucleic acid content, mainly ribonucleic acid (RNA) and its poor functionality (Otero et al., 2000). Therefore the isolation of yeast proteins is an attractive alternative for the utilization of yeast biomass through its use as emulsifying, gelling and foam stabilizing agents in food systems (Dzeziak, 1987b). An important number of works on yeast protein functionality (water holding capacity, viscosity, gelling and emulsifying properties) have been reported, mainly on *Saccharomyces cerevisiae* and other yeast, as *Kluyveromyces* (Barriga, Cooper, Idziak,

& Cameron, 1999; Cameron, Cooper, & Nufeld, 1988; Pacheco & Sgarbieri, 1998; Torabizadeh, Shojaosadati, & Tehrani, 1996; Vasallo, Puppo, Palazolo, Otero, Beress & Wagner, 2006). The aim of the procedures of fractionation generally employed is the isolation of yeast protein fractions with a reduced content of nucleic acids and with a high yield and solubility. In order to achieve a high yield and facilitate the cell rupture, the homogenization processes are usually performed in alkaline medium. This treatment produces yeast samples with non-defined composition of protein species and with an advanced degree of denaturation. In addition, the most of the fractionation methods of yeast components have been accomplished, focusing on the isolation of bioactive molecules such as β-glucans and enzymes.

From a functional viewpoint, a considerable work has been carried out on yeast protein isolates and isolated cell wall proteins (Pacheco & Sgarbieri, 1998; Vasallo et al., 2006). Nevertheless, there is scarce information and more investigation is needed to assess the potentialities of yeast fractions as food ingredients (Otero, Vasallo, Verdecia, Fernández, & Betancourt, 1996).

The present article focuses on a new protein fractionation from baker's yeast biomass. We proposed an inexpensive method of fractionation to obtain samples containing the totality of proteins of *Saccharomyces cerevisiae*. This procedure, based on differential centrifugation steps of insoluble components and subsequent precipitation of soluble fractions (in acidic medium and with acetone) was carried out in mild conditions in order to avoid protein denaturation. Moreover, the addition of a protease inhibitor before cell rupture was also evaluated. Chemical composition, thermal properties and surface behavior of obtained fractions were studied.

2. Materials and methods

2.1. Materials

Sample of *Saccharomyces cerevisiae* pressed bakers' yeast (Calsa, Lanús, Argentina) was purchased in a local supermarket. Chemical reagents of analytical grade were obtained from Merck and Sigma Chemical Co (St. Louis, MO, USA).

2.2. Preparation of yeast protein samples

The schematic process for the obtainment of yeast protein samples is shown in Fig.1. Commercial baker yeast (125 g, 30 g/100 g dry matter) were washed with 375 ml of 50 mmol/L sodium phosphate buffer pH 7.5. Then, the pellet (23 % dry matter) was resuspended in 375 ml of 50 mmol/L sodium phosphate buffer pH 7.5, with and without protease inhibitor (1 mmol/L phenylmethylsulfonyl fluoride, PMSF,). The cells were broken in a continuous glass bead grinder (Dyno-mill). Glass beads (diameter 0.5 mm) were previously washed with 1 mol/L hydrochloric acid and then with distilled water. Homogenization was made in the grinding chamber with 35 gr of yeast suspension and 15 g of glass beads, five times for 1 min. During the process, temperature was set at values below 10 °C. The rupture of yeast cell was confirmed by optical microscopy. Glass-free yeast homogenate was centrifuged at 5,000 rpm (3,840*g*, Beckman Coulter Avanti J25 centrifuge, JA14 rotor, Beckman Coulter, Fullerton, USA) for 15 min and the pellet (pellet 1) was washed with distilled water and freeze-dried (Thermovac freeze-dryer, Thermovac Industries Corp., USA). This fraction was called Fraction I and it was obtained as a flake-like brownish sample (**Fr I**). The turbid supernatant 1 was further centrifuged at 12,000 rpm (22,100*g*, Beckman Coulter Avanti J25 centrifuge, JA14 rotor, Beckman Coulter, Fullerton, USA) for 20 min, yielding a yellow precipitate labelled as Fraction II (**Fr II**), which was resuspended in distilled water and liophylised. The clean supernatant after the centrifugation (supernatant 2) was treated at 40° C for 1 h under magnetic stirring and NaCl was added (3 g/100 mL). Then, this supernatant was adjusted to pH 4.5 with HCl and subsequently centrifuged at 22,100*g* for 20 min. The precipitate (pellet 3) was washed with water and freeze-dried (Fraction III, **Fr III**). The supernatant 3 was treated with cold acetone (-20° C) at 50 % v/v. The precipitation was performed in a water-ice bath and the addition of acetone was carefully performed to avoid temperature increase. The precipitate (pellet 4) was washed and resolubilized in water, and freeze-dried. This fraction was named Fraction IV (**Fr IV**). Fr III and Fr IV were obtained as pale yellow powders.

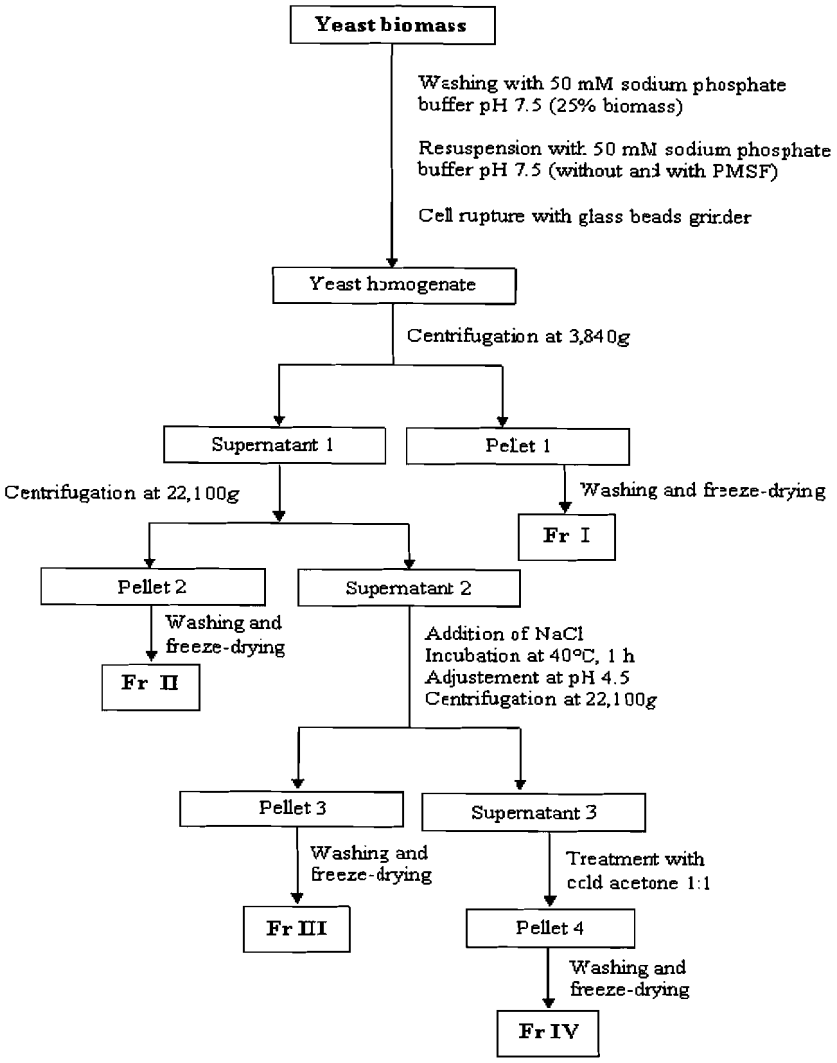

Fig. 1. Schematic process for the fractionation of yeast protein samples from *Saccharomyces cerevisiae.*

2.3. Determination of RNA

Nucleic acid content was determined by hot perchloric acid extraction by following the experimental procedure of Rut (1973).

2.4. Determination of crude and corrected protein

Protein content was determined by Kjeldahl method. Kjeldahl digestions were carried out in micro-Kjeldahl flasks with 50 ml calibration marks. Total Nitrogen (TN), which includes nitrogen from protein, peptides, free amino acids and nucleic acids, was determined in the digested samples by the colorimetric method of Nkonge & Murray-

Ballance (1982). Standard nitrogen solutions were prepared by using oven-dried (3 h at 105° C) ammonium sulphate. Blank digest were prepared in an identical manner by using only catalyst and sulphuric acid. Nucleic acid nitrogen (NAN) was determined from nucleic acid content by using the factor 5.7, which take into account the mean composition of purines and pyrimidines. Crude protein and corrected protein content values were determined as TN×6.25 and (TN - NAN)×6.25, respectively.

2.5. Determination of soluble protein

Aqueous dispersions were prepared by dissolving the lyophilized samples in 10 mM sodium phosphate buffer, pH 7.0 (10 mg sample/mL) for at least 2 h under magnetic stirring to ensure the complete dispersion of the sample. A high speed blender (Ultraturrax T-25, SN-25-10G dispersing tool, IKA Labortechnik, GmbH & Co, Staufen, Germany) at 20,000 rpm for 30 s was further used to obtain a fine dispersion. Then, the samples were centrifuged at 10,000*g* for 15 min (IEC Centra MP4R centrifuge, International Equipment Company, USA). The protein concentration in the supernatants was determined by the Biuret method (Gornall, Bardawill, & David, 1949) using bovine serum albumin was as standard protein. Solubility was expressed as grams of soluble protein/100 g dry sample.

2.6. Determination of carbohydrates

Total carbohydrate was measured by phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) with 5.0 g/100 mL phenol and assayed at 490 nm. Glucose was utilized as standard.

2.7. Determination of lipids

Total fat were determined by Soxhlet extraction using *n*-hexane and methanol as organic solvents. Taking these two solvents of strongly different polarity allows the extraction of non-polar parts as well as more polar components of the lipids. The mixture *n*-hexane-methanol assures total extraction of fat, as was reported by Freimund, Sauter, Käppeli, & Dutler (2003).

2.8. Determination of dry matter and moisture

Dry matter and moisture was determined by heating the samples at 105° C to constant weight.

2.9. Determination of adsorption behavior at air-water interface

To evaluate the surface properties of protein samples at the air/water interface, the supernatants obtained by centrifugation of fine aqueous dispersions were utilized. In all cases, the supernatants were diluted in order to obtain a soluble protein concentration of 0.05 g/100 mL. The surface tension (γ, in mN/m) was measured at 20° C according to dynamic method using an automated drop volume tensiometer Lauda TVT 2 (Lauda-Königshofen, GmbH & Co., Germany). The principle of the drop volume technique is to measure the volume (or weight) of a drop detaching from a capillary with a circular cross-section. The dosing rate allows the control of the formation of drops in a drop time range of 0.3 s up to 30 min per drop. From the volume of the drop and the capillary diameter, γ can be calculated by using the Gauss-Laplace equation (Miller, Hoffmann, Hartmann, Schano & Halbig, 1992). As a result of these experiments, the γ-$t^{1/2}$ curves were recorded. The initial slope ($\Delta\gamma/\Delta t^{1/2}$) was calculated from the linear portion of the γ vs $t^{1/2}$ curve. These values are in aggrement with the rate of absorption, especially in the first step of process. Assays were conducted in duplicate.

2.10. Electrophoresis

Samples were extracted during 2 h at 20° C with a buffer containing 1.5 mol/L Trizma, 0.4 g/100 mL SDS, pH 8.0 in the presence of 5 g/100 mL of ß-mercaptoethanol. Protein dispersions were diluted with an equal volume of a pH 6.8 buffer 0.125 mol/L Tris-HCl, 0.1 g/100 mL SDS, 40% v/v glycerol and 0.05 g/100 mL bromophenol blue. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed according to the method of Laemmli (1970) modified by Puppo et al. (1995) using a continuous gel of 10 % in polyacrylamide. A continuous dissociating buffer system was used, containing 0.375 mol/L Tris-HCl, pH 8.8, and 0.1 g/100 mL SDS for the separating gel and 0.025 mol/L Tris-HCl, 0.192 M glycine, and 0.1 g/100 mL SDS, pH 8.3, for the running buffer. The gel slabs were fixed and stained overnight by addition of 0.1 g/100 mL

Coomasie Blue R-250 solution (40 % v/v methanol, 10 % v/v acetic acid). Electrophoresis was carried out at constant voltage (200 V). Low MW markers (Pharmacia calibration kit, Pharmacia Biotech, Uppsala, Sweden) used included: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), Ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa); α-Lactalbumin (14.4 kDa). Molecular weight of different polypeptides were achieved by analysis of bands using a Bio Rad Molecular Analyst Program (Bio Rad Laboratories Inc., USA).

2.11. Differential scanning calorimetry

Samples (10-15 mg) of 25 g/100 mL dispersions in water were hermetically sealed in aluminium pans and heated from 30 to 100° C at heating rate of 10° C/min (DSC Polymer Rheometric Scientific, Piscataway, NJ, USA). A double empty pan was used as reference. Denaturation enthalpies and peak temperaure (T_p) were drawn from the corresponding thermograms (Software Plus V5.41, USA). Enthalpies values (ΔH) were expressed as J/g protein, taking into account the dry weight (determined by perforating the pans and heating overnight at 105° C) (Wagner & Añón, 1990) and the protein content of sample. All assays were conducted in duplicate.

Preparation and Stability of o/w emulsions

The emulsions were prepared by homogenization of 10 mL of a sample dispersion (10 mg/mL, 0.01 M sodium phosphate buffer, pH 7.0) and 10 mL of corn oil using an Ultraturrax (T-25, S25N10G device, IKA Labortechnik, Karlsruhe, Germany) at 20.000 rpm for 30 s. at 25° C.

Emulsion stability agains creaming (creaming stability) was determined at 25° C in a Vertical Scan Analyzer (QuickScan, Beckman-Coulter, USA). Samples were put in a cylindrical glass measurement cell and the Backscattering (BS%) profiles were studied each minute during one hour as a function of the sample height (total height 60 mm) . Initial Backscattering (BS1) values were determined from initial profile of emulsions (t=1 min) as the mean value through the tube length. Creaming kinetic was followed by measuring the mean values of BS% as a function of time in the bottom zone of the measurement cell (zone 5-7 mm). Relative volume of cream phase at 60 min (Vr), respect to total emulsion volume, was estimated from the BS % profiles.

3. Results and discussion

According to procedure of yeast protein fractionation previously described in Fig. 1, four samples (Fr I, Fr II, Fr III and Fr IV) were obtained. The amount of each sample expressed as percentage ratio (R %) is shown in Table 1. Fr I was significantly more abundant than other fractions (R % = 40-50). The comparison of R % values, obtained from the assays performed without and with PMSF, showed that the presence of protease inhibitor increased the R % value of Fr I, in detriment of the other fractions.

According to the fractionation procedure (Fig. 1), the sample Fr I, which is the fraction of highest density after cell rupture, is supposed to be mainly cell wall debris. On the other hand, Fr II, could correspond to insoluble cell and organelle membrane components which are responsible of turbitidy of supernatant at 3,840*g* (supernatant 1) and therefore required a higher centrifugal force to sediment (22,100*g*).

Samples Fr III and Fr IV, resultant from the two first step of centrifugation, are composed mainly by cytoplasm soluble proteins. When the supernatant 2 is adjusted at pH 4.5 by acid, Fr III is obtained, so it is composed by acid insoluble proteins. The proteins that remain soluble at pH 4.5, are further precipitated with acetone and constitute the fraction IV (Fr IV). To corroborate that the obtained samples are different and contains the above mentioned cell components, a composition study was carried out. Table 2 shows the content in protein, nucleic acids, carbohydrates and lipids in comparison with the whole cells. In the absence of PMSF, Fr I showed an remarkable high content of carbohydrates (> 70 g/100 g), due to the presence of cell wall polysaccharides, mainly glucans and mannans (Walker, 1998; Kapteyn, Van den Ende and Klis, 1999).

The pronounced enrichment of polysaccharide content of Fr I respect to whole cells reveals that this fraction is mainly composed by cell wall debris. Freimund et al. (2003) studied the compositions of two yeast cell walls fractions (YCW) from different yeast factories; their content of polysaccharides was very variable (39-56 g/100 g) whereas the lipid and protein content were in close agreement (11-13 and 22-29 g/100 g, respectively). Fr I showed only a similar content of protein respect to YCW fractions, whereas the composition of carbohydrates and lipids exhibited clear differences (Table 2).

Table 1: Percentage (R %) of yeast fractions obtained by cell rupture and fractionation in the absence (+) and presence (-) of protease inhibitor (phenylmethylsulfonil fluoride,

PMSF). The ratio for each fraction was determined as: R (%) = [weight of fraction (g) / weight of all fractions (g)] ×100

Sample	PMSF	R (%)
Fr I	+	51.09 ± 1.05
Fr II	+	17.31 ± 0.95
Fr III	+	17.71 ± 0.89
Fr IV	+	13.89 ± 0.75
Fr I	-	42.95 ± 1.17
Fr II	-	20.85 ± 1.01
Fr III	-	19.33 ± 0.84
Fr IV	-	16.87 ± 0.85

All the data were expressed as the mean and S.D. of duplicate measurements

The reasons for these results are likely due to growth and processing conditions. The molecular architecture of the cell wall is not constant. Cells could exhibit significant differences in cell wall composition and structure as a function of environmental conditions, such as temperature and pH. In the proposed fractionation procedure, a slighlty alkaline medium (pH 7.5) and room temperature were used. Therefore, Fr I would contain the main components of the cell wall of *Saccharomyces cerevisiae*: cell wall proteins (CWPs, phosphorylated mannoproteins), β-(1,6)-glucan and β-(1,3)-glucan. These components are all interconected by covalents bonds (Walker, 1998; Kapteyn et al., 1999). However, some of these mannoproteins are solubilized during the preparation of aqueous dispersions (soluble protein content ≈ 8 g/100 g, Table 2).

On the other hand, nuclei acid content of Fr I was significantly lower than the other fractions and whole yeast (2.32 g/100 g), which was within the range normally found in food products like organ meats, seafoods, lentils and beans (Pacheco & Sgarbieri, 1998). In this fraction, nucleic acids are a contamination. Fr II has similar crude and corrected protein contents than whole cells, while lipid content was the highest among all fractions. These protein would be present in both plasmatic and organelle membranes. In addition, the soluble protein content of Fr II, determined by Biuret method, was approximately four times higher than that corresponding to Fr I.

Table 2: Chemical composition of whole yeast and its protein fractions. With exception of moisture, all values are expressed in dry basis.

Sample		Crude Protein (%)	Corrected protein (%)	Soluble protein (%)	Nucleic acids (%)	Total carbohydrates (%)	Lipids [a] (%)	Moisture (%)
Whole yeast		48.1 ± 0.9	40.5 ± 0.9	n.d.	6.9 ± 0.1	40.2 ± 1.4	7.0 ± 1.2	69.5 ± 0.1
Fr I	with PMSF	22.6 ± 1.4	20.0 ± 1.4	7.7 ± 0.2	2.3 ± 0.1	72.2 ± 2.8	5.1 ± 0.6	9.1 ± 1.0
Fr II		48.8 ± 2.2	42.0 ± 2.1	27.4 ± 0.4	6.2 ± 0.1	9.7 ± 0.5	29.4 ± 1.7	13.1 ± 0.5
Fr III		64.3 ± 2.1	58.8 ± 2.0	14.5 ± 0.3	5.0 ± 0.1	7.5 ± 0.5	Negligible	6.5 ± 0.4
Fr IV		65.0 ± 2.2	54.1 ± 2.1	54.9 ± 0.3	10.0 ± 0.1	15.6 ± 3.0	Negligible	11.1 ± 0.1
Fr I	without PMSF	19.7 ± 0.3	18.2 ± 0.3	10.0 ± 0.3	1.4 ± 0.0	77.4 ± 3.3	n.d.	9.2 ± 0.1
Fr II		45.1 ± 3.5	39.4 ± 3.4	37.2 ± 0.4	5.2 ± 0.1	11.8 ± 0.9	n.d.	12.2 ± 0.6
Fr III		68.3 ± 3.3	60.9 ± 3.3	14.9 ± 0.2	6.8 ± 0.1	4.4 ± 0.7	n.d.	6.7 ± 0.2
Fr IV		55.5 ± 1.1	45.8 ± 1.2	50.6 ± 0.6	8.8 ± 0.2	15.5 ± 1.8	n.d.	10.8 ± 0.9

All the data were expressed as the mean and S.D. of triplicate measurements. [a] n.d. = non determined

The presence of lipid components, phospholipids and sterols, which are effectively extracted by a mixture of two organic solvent of different polarity (*n*-hexane-methanol) would explain the high fat content obtained in this fraction (≈ 30 g/100 g, Table 2). The experimental fractionation procedure (see Figure 1) included two steps of centrifugation at different centrifugal force and hence, the separation is based on size and density. It is probable that smaller cell wall fragments are also present in Fr II. Carbohydrate and nucleic acid contents of Fr II were approximately 10 and 6 g/100 g, respectively (Table 2). Although it is possible that cell wall polysaccharides are present in this sample, the contribution of ribose residues of nucleic acid to total carbohydrate content must also be considered.

As was previoulsy mentioned, a limiting factor of utilization of yeast biomass is its high nucleic acid content, basically ribonucleic acid. Some reagents and techniques used for the isolation of yeast protein fractions with low nucleic acid content may cause alteration in protein structure and have been scarcely used in treatment for a variety of reasons that range from the impracticability of the operation scale needed, to potential toxicity of the solvent used (Guzman-Juárez, 1982, Pacheco & Sgarbieri, 1998). In the present work, the

clean supernatant after the second step of centrifugation was treated in mild conditions, at 40° C with the addition of NaCl for 1 h in order to activate the endogenous ribonuclease. Yeast RNases exhibit optimal temperatures very close to 50° C. Otero et al (2000) reported that in *Saccharomyces cerevisiae* protein samples, thermal denaturation starts below 50° C. Therefore, a lower incubation temperature (40° C) was chosen to preserve native structure of proteins. Fr III, obtained by acid-precipitation of this supernatant is supposed to be composed mainly by cytoplasm proteins, especially nucleoproteins, which are released to the bulk of suspension once the external structures are disrupted. According to Table 2, this fraction exhibited a relative protein enrichment respect to whole cells, similar to yeast protein concentrates. These concentrates are obtained by isoelectric precipitation of soluble fraction of yeast homogenate after a centrifugation step. Pacheco & Sgarbieri (1998) reported that these protein concentrates are composed mainly by cytoplasm enzymes and nucleoproteins and their RNA was extremely high (> 15 g/100 g on dry basis). Fr III, however, exhibited nucleic acid content significantly lower (≈ 5 g/100 g), although it was only slightly lower than RNA in intact cells. The soluble protein content of Fr III is close to 15 g/100 g (Table 2). A value slightly lower than what reported Pacheco & Sgarbieri (1998) for yeast protein concentrates in a previous paper. Fr IV is a fraction composed by cytoplasm proteins soluble at acid pH (pH 4.5). This fraction was the most easily dispersible in the buffer during the preparation of aqueous dispersions; this fact became evident since a similar content of corrected and soluble protein was observed. In addition, this fraction exhibited the highest content of nucleic acid among all fractions. Then, it is highly probable that proteins were also bound to nucleic acid. According to previously mentioned results, a more drastic treatment to reduce nuclei acid content is required for Fr III and Fr IV. In both samples, (Fr III and Fr IV) carbohydrate content was probably overestimated due to the presence of nucleic acids. Moreover, no fat was practically extracted when these samples were treated with a mixture of *n*-hexane-methanol (Table 2). When cell rupture was performed in absence on PMSF, a slight decrease of crude and corrected protein was observed in Fr I and Fr II, while soluble proteins, determined by Biuret method, and was higher in these fractions, mainly in Fr II. This fact could be attributed to the action of endogenous proteases. Moreover, both fractions showed a slight increase in the carbohydrate composition, whereas the nucleic acid contents exhibit a small reduction. Among the samples obtained by precipitation during the fractionation procedure, the absence of protease inhibitor affected the crude and corrected protein content of Fr IV, showing in both cases a pronounced decrease. In contrast, the protein

content of Fr III was not significantly changed with respect to the sample obtained by fractionation in the presence of PMSF. In this sample, a small increase of nuclei acid content was observed (Table 2).

SDS-PAGE patterns showed that all samples have a different polypeptide composition (Fig. 2). As was mentioned above, an exogenous serin protease inhibitor (PMSF) was added prior to cell rupture in order to study the effect of their addition on the characteristics of samples obtained.

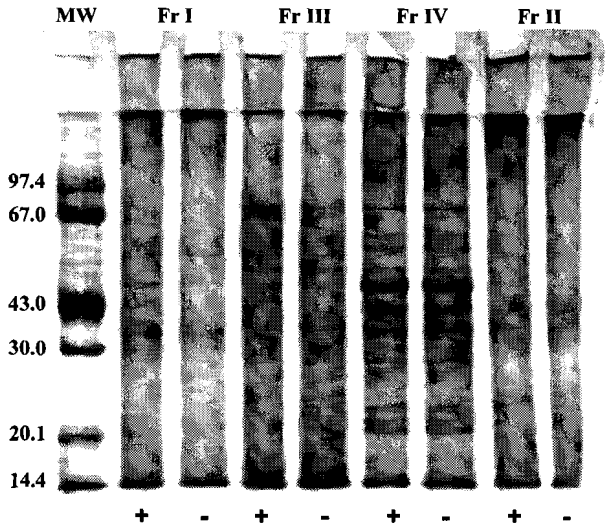

Fig. 2. SDS-PAGE profiles of yeast protein samples in reducing conditions. The simbols (+) and (-) are related with the presence and absence of protease inhibitor (phenylmethylsulfonyl fluoride, PMSF) during cell rupture. Molecular weights are expressed in kDa.

The patterns did not exhibit important differences among samples obtained in absence or presence of PMSF. It seem to be that in PMSF absence, some bands on SDS-PAGE patterns of Fr I and Fr II are weaker than those corresponding to the experiments with protease inhibitor. A wide variety of proteases is present in yeast cells. The major proteases are vacuolar proteases A, B and C, this latter also named carboxypeptidase Y (Jazwinsky, 1990, Walker, 1998). Protease A is an acidic endoproteinase that is largely inactive below pH 6, and therefore it is inactivated during the acid precipitation (pH 4.5) in Fr III isolation. Although PMSF is quite effective with proteases B and C, fortunately these enzymes (and also protease A) are found as complexes with their natural inhibitors in yeast homogenates around neutral pH (Jazwinsky, 1990); in this work, we carried out the cell rupture and centrifugation steps at pH 7.5. Therefore, it is highly probable that proteolysis during the fractionation procedure was not sufficiently extensive. Fr I showed a main band at 35 kDa, which is only slightly less intense for the sample obtained without PMSF (Fig.

2). The presence of high molecular species that could not penetrate the separating gel was also evident; it is possible that these proteins are linked with cell wall polisaccharides. Mrsa, Seidl, Gentzshm & Tanner (1997) were able to identify approximately 20 CWPs. Nine of them were extractable with hot SDS-β-mercaptoethanol, where 11 of them remained as insoluble protein. Two main classes of CWPs (GPI-CWPs and Pir-CWPs) can be distinguished in detergent-soluble fraction, which are extractable with β-glucanase (GPI-CWPs) and by treatment in mild alkali condition or by β-glucanase digestion (Pir-CWPs). When isolated cell walls are digested with a crude β1,6-glucanase and liberated proteins are separated by SDS-PAGE and analyzed by western blotting a characteristic pattern of four β-glucosylated proteins appears with relative molecular masses of approximately 60, 80, 145 and 220 kDa (Kapteyn et al., 1999). In this work, Fr I were obtained in the fist step of centrifugation after a rupture process and no enzymatic digestion was carried out. This result explain the marked differences in the electrophoretic pattern with regard to those reported by Kapteyn et al. (1999) in a previous work. Respect to Fr II sample, an important aggregation degree is observed; it is important to take into account that the membrane proteins form complexes with unsaturated polar lipids. A possible lipid oxidation during the storage of this sample could be responsible of an additional protein polymerization. This fact results evident due to the presence of a broad band in the upper part of separating gel: these species have molecular masses significantly higher than 97.4 KDa. Fr III and Fr IV are composed by proteins with a greater amount of polypeptides that penetrate to the separating gel. Fr III shows main bands at 78, 54, 48 and 37 KDa. Moreover, this sample showed an important content of small polypeptides (molecular mass < 14 kDa). On the other hand, Fr IV exhibit a main band at 50 KDa and other minor species of 78, 65, 60, 47, 41, 30, 26, 22 and 20 KDa. The presence of small polypeptides at molecular masses < 14 kDa was also evident for this sample. The effect of PMSF addition during the fractionation procedure was not clearly observed in the electrophoretic patterns of Fr III and Fr IV: no noticeable differences were observed in protein bands in absence or presence of protease inhibitor.

The thermal analysis of yeast and its derived protein was carried out. The corresponding thermograms are shown in Fig. 3; peak temperatures (T_p) and enthalpy values (ΔH) of endothermic peaks are showed in Table 3. Thermograms of whole cells exhibited two endothermic peaks: peak I to 66.1 and peak II at 74.4 °C (Fig. 3 a); nevertheless, the process of desnaturalization starts to approximately 50-55 °C. Then, in a first

approximation, fresh and active yeast sample are composed by two protein fractions with different thermal stability.

Table 3: Peak temperatures (T_p) and denaturation enthalpies (ΔH) of whole yeast and its fractions. These samples were obtained by cell rupture and fractionation with (+) and without (-) phenylmethylsulfonyl fluoride (PMSF) (See Figure 1).

Sample		T_p (°C)		ΔH (J/g protein)
		Peak I	Peak II	
Whole yeast		66.1 ± 0.2	74.4 ± 0.1	20.5 ± 1,6
Fraction	PMSF	Unique peak		
I	+	57.6 ± 0.4		27.1 ± 0.9
II	+	63.1 ± 0.7		46.0 ± 3.7
III	+	59.1 ± 0.2		10.7 ± 1.0
IV	+	74.1 ± 0.3		15.1 ± 0.8
I	-	No peak		0
II	-	63.4 ± 0.6		17.9 ± 0.5
III	-	57.2 ± 0.4		16.1 ± 0.2
IV	-	73.7 ± 0.1		16.1 ± 0.3

All the data were expressed as the mean and S.D. of duplicate measurements

In a previous work (Otero et al. 2000), a main peak at 66.6 °C was observed in DSC thermograms of active dry yeast; minor peaks at temperatures lower than 60° C and higher than 70 °C were also observed probably due to conformational changes during the drying process. Entalphy value of protein denaturation in fresh yeast (20.5 J/g) was higher than those obtained in dried samples (near 14-16 J/g, Otero et al., 2000, 2002), indicating a lower degree of denaturation in non-dried samples. DSC thermograms of samples Fr I - Fr IV obtained during the fractionation in presence of PMSF exhibited a unique endotherm with different T_p and ΔH values (Fig. 3 b, Table 3).

These results would indicate that proteins present in Fr II and Fr IV are more stable that those of Fr I and Fr III. At first, it would have a partial coincidence between fractions T_p and the T_p of peaks I and II of the fresh yeast sample. Although the native structure has not been appreciably affected, when proteins are isolated from their natural environment, a shift of denaturation peak towards smaller temperatures can occur. This result appears to

be related to the protective environment of intact cells, which is loss during the rupture process (Huang & Kinsella, 1986).

If we consider that protein denaturation in whole cells (peak I) starts at 50-55° C, denaturation of Fr I, Fr II and Fr III proteins would contribute with peak I, while those of Fr IV will contribute to peak II of yeast thermogram.

On the other hand, the endotherms of Fr I and Fr III would correspond to the weak peak at temperatures below 60° C in dried yeast thermogram. This fact indicates that the process of isolation and drying have increased the thermal sensitivity of proteins that constitute these fractions. According to Hromádková, Ebringerová, Sasinková, Šandula, Hříbolová & Omelková (2003), the drying by lyophilisation affects the structure of cell wall β-glucans; thus, the same process could affect the integral structure of β-glucans and mannoproteins of Fr I.

Regarding to denaturation enthalpy, it can be seen that this value is higher in Fr I and Fr II, which are constituted by the external proteins of the cellular structure and that form complexes with polisaccharides or lipids. Fr III, composed mainly by cytoplasm proteins and nucleoproteins, exhibited T_p and ΔH values more similar to those of isolated yeast proteins informed previously (Otero el al., 2000). Nevertheless, these isolated prepared from yeast according to the described procedure (rupture and solubilization at alkaline pH followed by acid precipitation and drying) are mainly constituted by cytoplasm proteins and nucleoproteins, but contaminated with soluble proteins from cell wall and membrane. Denaturation pattern produced an unique endothermic peak with T_p below 60°C and ΔH lower than 4 J/g (Otero el al., 2000), indicating a marked structural change by isolation process.

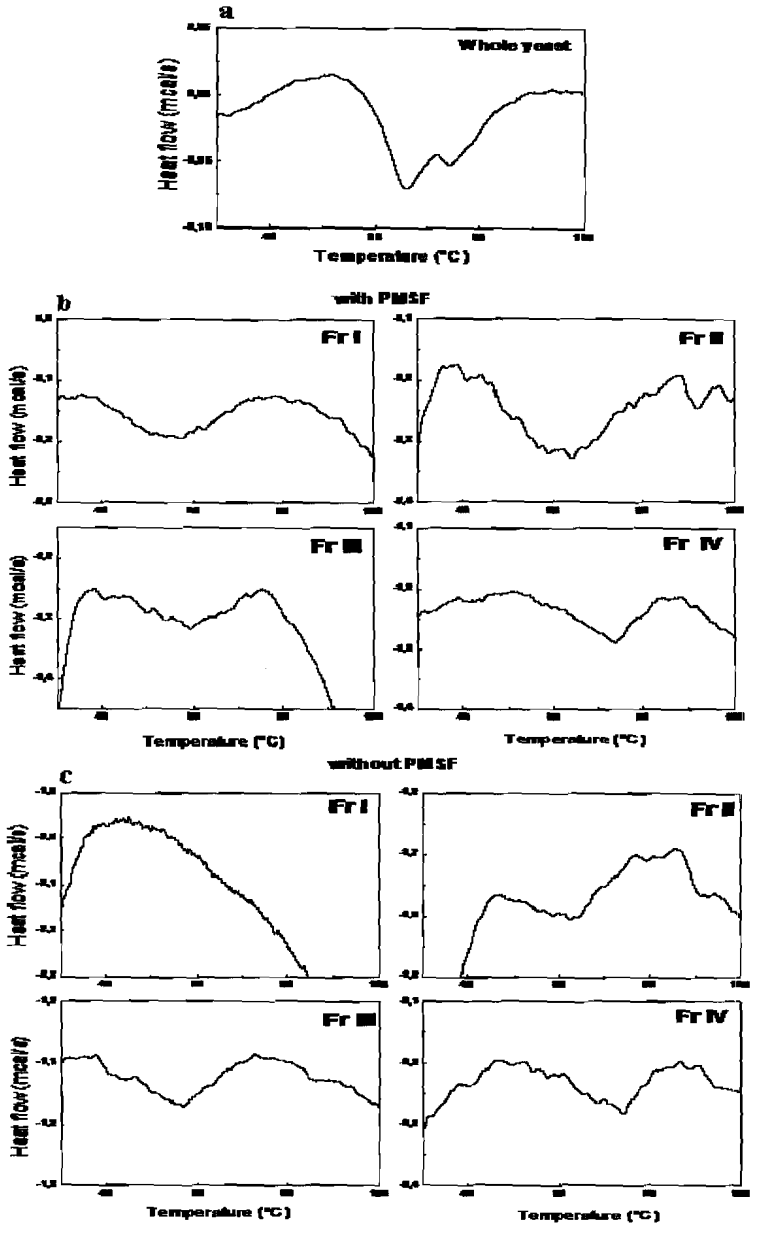

Fig. 3. DSC thermograms of intact cells of *Saccharomyces cerevisiae* (baker's yeast) and protein samples obtained by fractionation of yeast homogenates. Cell rupture was performed without and with phenylmethylsulfonyl fluoride (PMSF). The experimental fractionation procedure are shown in Figure 1.

When fractions were obtained without the presence of protease inhibitor, Fr I did not give any DSC endotherm whereas for Fr II it is seen very reduced the denaturation entalphy with respect to the same sample obtained with PMSF. (Fig. 3 c, Table 3). It suggests that, although not detectable by SDS-PAGE, some structural change in absence of PMSF must have affected the stability of cell wall proteins and, in a smaller degree the membrane proteins. The activity of enzymes non-inhibited by PMSF (β-glucanases, lipases, chitinases, etc.) could have had some relation with these changes. On the other hand,

thermal stability of cytoplasm proteins and nucleoproteins exhibited no effect by the absence of protein inhibitor.

Knowing the composition and the thermal stability the obtained fractions, it was come to a study of their superficial behavior. The kinetic adsorption at the air/water interface of all fractions was followed by a dynamic method, by using a drop volume tensiometer. These assays were performed at the same soluble protein concentration (0.05 % w/v) and pH. In the absence of protease inhibitor, the more efficient samples for decreasing surface tension were Fr I and Fr II (Fig. 4 a).

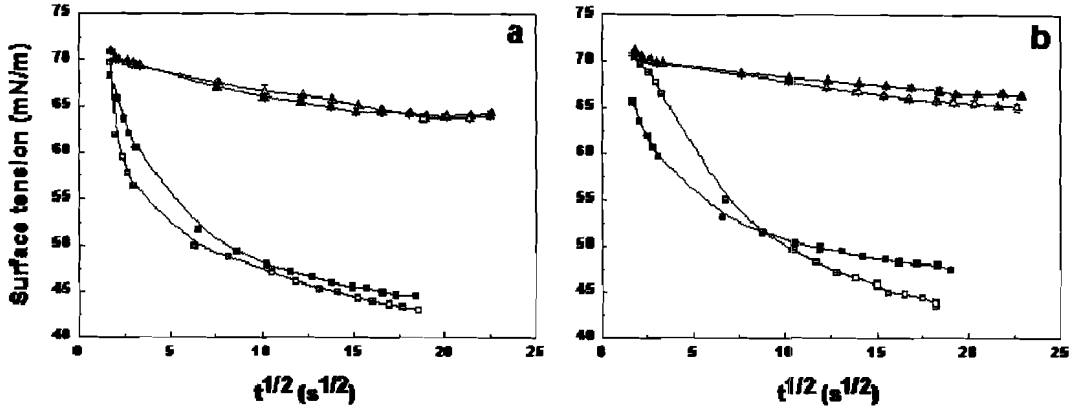

Fig. 4. Adsorption kinetics of soluble proteins of yeast protein samples at air/water interface. These samples, Fr I (■), Fr II (□), Fr III (▲) and Fr (△), were obtained by fractionation in the absence (a) and presence (b) of protease inhibitor (phenylmethylsulfonyl fluoride, PMSF). The solubilization and measurement buffer was 10 mmol/L sodium phosphate buffer (pH 7.0) and the protein concentration was 0.05 mg/mL.

These results suggest the presence of proteins with high ability to adsorb at air/water interface. For Fr I, the wall mannoproteins solubilized during the preparation of aqueous dispersions would behave as a effective tensioactive agent, due to their faster diffusion and anchorage to the interface. The high surface and emulsifying activity of mannoprotein samples, obtained in different conditions, were reported in previous works (Torabizadeh et al., 1996; Barriga et al., 1999; Vasallo et al., 2006). For Fr II, this behavior is attributed to phospholipids or protein-phospholipid complexes presente in membranes. In contrast, Fr III and Fr IV, exhibited a poor and similar surface activity, as a consequence of the presence of globular cytoplasm proteins and nucleoproteins that diffuse more slowly to the interface. The precipitacition of these proteins in acid medium or acetone could negatively affect their surface properties. The initial slopes ($\Delta\gamma/\Delta t^{1/2}$) values for Fr I, II, III and IV were 5.68, 5.71, 0.53 and 0.43 mN/m.s$^{1/2}$, respectively (Fig. 5 a).

Moreover, results obtained in the presence of PMSF showed that addition of protease inhibitor before cell disruption had a evident negative effect on surface activity, mainly in the first step of adsorption process (Fig. 4 b). The decrease percentages in the $\Delta\gamma/\Delta t^{1/2}$ values for Fr I, Fr II, Fr III and Fr IV were 25.2, 53.2, 56.6 and 32.5 %, respectively (Fig. 5 b). The presence of protease inhibitor during the fractionation avoids the generation of soluble peptides of lower molecular weight, which diffuse fastly to the air/water interface. Nevertheless, the presence of these peptides was not evident in the electrophoretic patterns (Fig. 2). The tendency observed in surface activity of samples was similar respect to those observed without PMSF. The samples obtained by differential centrifugation steps (Fr I and Fr II) showed a better surface behavior than Fr III and Fr IV.

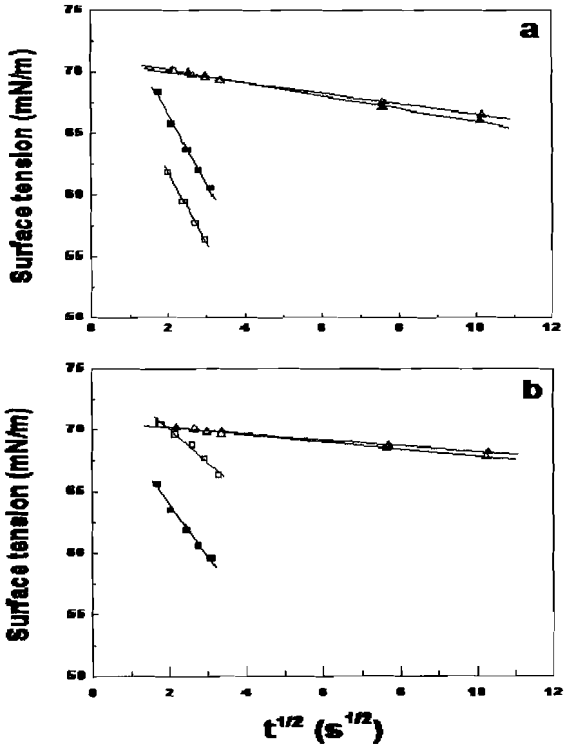

Fig. 5. Initial slopes ($\Delta\gamma/\Delta t^{1/2}$) values as a function of $t^{1/2}$ for yeast protein fractions obtained by cell rupture and fractionation without (a) and with (b) phenylmethylsulfonyl fluoride. Samples: Fr I (■), Fr II (□), Fr III (▲) and Fr (△).The parameter $\Delta\gamma/\Delta t^{1/2}$ is associated with the first step of adsorption process. The solubilization and measurement buffer was 10 mmol/L sodium phosphate buffer (pH 7.0) and the protein concentration was 0.05 mg/mL.

Micrograph of emulsion prepared with samples FI to FIV are showhed in Figure 6. he emulsion corresponding to F II was composed with more little droplets.

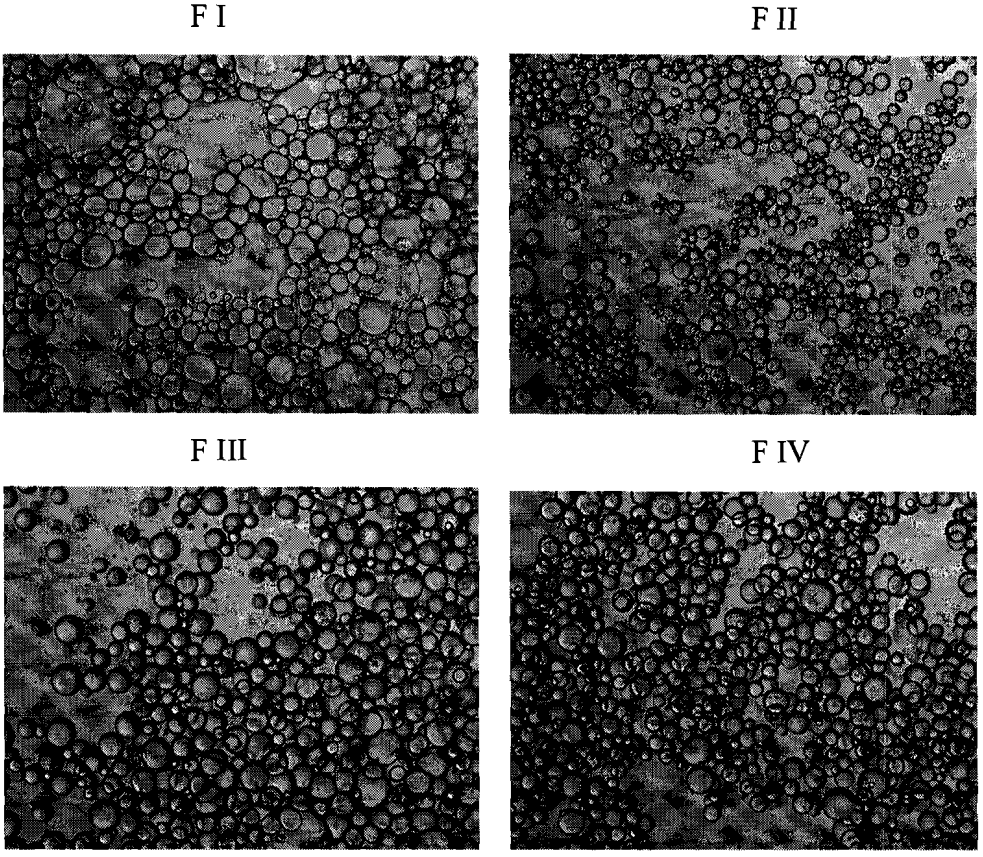

Figure 6: Microstructure of emulsions. Magnification: 100 X.

To determine the stability of emulsions prepared with different samples, the back scattering (BS %) profiles were analyzed according Palazolo et al. (2004). High value of BS initial for Fr I correlates with the small particles. Creaming kinetic was analyzed in the lower part of tube by means of BS% decrease as a function of time (Figure 7). Emulsion from Fr II showed a high creaming stability, which correspond with their composition in phospholipids, the higher tensioactivity and lower diameter of droplets. In this regard, drop size seems to be the most important factor concerning to stability.

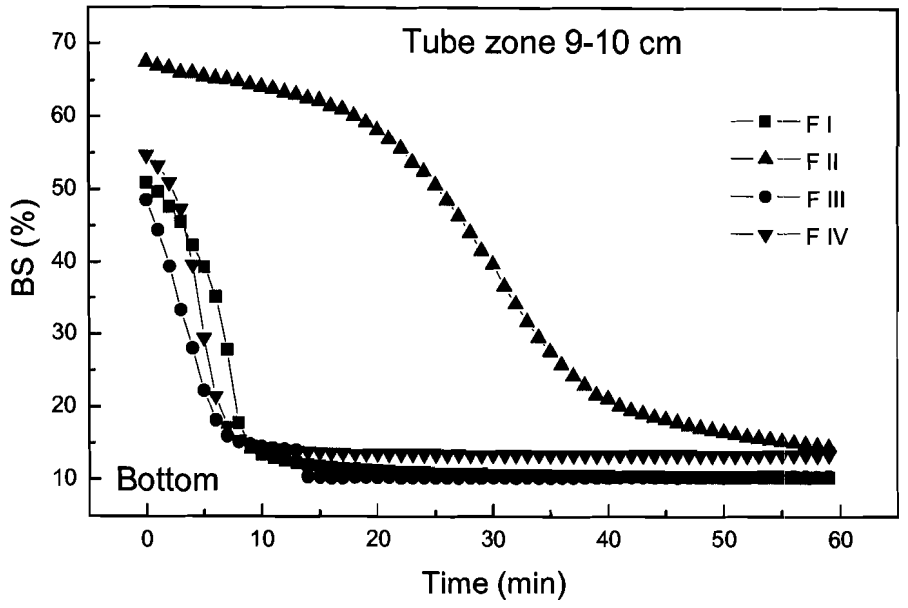

Figure 7: Creaming process of emulsions prepared with different yeast fractions. To follow the destabilization, BS variation in the bottom zone of tube (9-10 mm) was analyzed.

FI, composed by wall proteins with acceptable surface properties, produce an emulsion with lower creaming stability, perharps due to desestabilizing effect of presence of insoluble polysaccharides.

4. Conclusions

The fractionating method for yeast biomass proposed herein is easy and inexpensive and it is carried out in mild conditions. The corresponding yeast protein fractions are constituted by proteins that proceed from distinct parts of the yeast cells. Hence, they also exhibit thermal, surface properties and a chemical composition markedly differents. The easiness of proposed method would make possible the scaling up in order to obtain yeast protein fractions that could have a potential application as functional ingredients.

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