Bull. Fac. Agric., Cairo Univ., 60 (2009):74-85.

PRODUCTION OF BIOMASS FROM WHEY PERMEATE AND STARCH HYDROLYSATES BY *Saccharomyces cerevisiae* AND *Kluyveromyces marxianus*

(Received: 8.9.2008)

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ABSTRACT

Three local strains of *Saccharomyces cerevisiae* (*Sc 1*, *Sc 2* and *Sc 3*) and one strain of *Kluyveromyces marxianus* were used as pure or mixed cultures in a commercial medium, in order to compare their kinetic parameters and fermentation patterns. It was found that *S. cerevisiae Sc 3* had the highest maximum specific growth rates on glucose (0.32 h^{-1}) and on ethanol (0.11h^{-1}). The yields of biomasses on glucose and on ethanol were the most important. A low yield of ethanol on glucose 0.33 (g/g) was obtained. Kinetic studies of continuous production of biomass from a mixture of whey permeate and starch hydrolysates using mixed culture of *Saccharomyces cerevisiae Sc 3* and *Kluyveromyces marxianus* were performed. In the course of continuous culture on mixed substrate of whey permeate and starch hydrolysates "A", *Kluyveromyces marxianus* overgrew *Saccharomyces cerevisiae*. Whereas in the course of continuous culture on mixed substrate of whey permeate and starch hydrolysates "B" there was a stabilization of the coculture (*Saccharomyces cerevisiae Sc 3* and *Kluyveromyces marxianus*).

Key words: *biomass production, Kluyveromyces marxianus, Saccharomyces cerevisiae, starch hydrolysates, whey permeate*

1. INTRODUCTION

The rapid increase in the worldwide population and the limited reserves in the conventional plant proteins, have stimulate research for new protein sources.

It is possible to produce proteins in the industrial scale from unicellular organisms, notably from yeast. This way of production presents triple interest: 1) it is independent from the climate nature or the soil quality, 2) it has rapid production rate and a continuous production process can be used and 3) the substrates for the fermentation is abounding and not very costly because these basically are by-products of various industries: starches, molasses of sugar beet, sulfite liqueurs from paper mill, lactoserum of dairy industries. Among the main agro-industrial wastes, whey and starch are of prime importance (Compango *et al.*, 1995). Whey waste is a major problem for the dairy industry (Ghaly and Kamal, 2004). Finding alternative means to reduce its pollution potential and produce high value-added bio-ingredients has been attempted by many researchers. *Kluyveromyces marxianus var marxianus* is a dairy yeast that produces beta-galactosidase, allowing for whey fermentation (Lukondeh *et al.*, 2005, Schultz *et al.*, 2006). Also, *K. marxianus* has been proposed as a source of oligonucleotides, oligosaccharides, and oligopeptides (Belem and Lee, 1998). Paul *et al.* (2002) determined the nutritional profile of food yeast *K. fragilis* grown on deproteinized whey supplemented with 0.8 % diammonium hydrogen phosphate and 10 ppm indole-3-acetic acid. Starch is present in a number of reserve organs of plants, cereals such as wheat, corn, barley and the tubercles such as potato and manioc. The yeast *Saccharomyces cerevisiae* could not utilize directly the starch. For this reason the starch should be hydrolyzed to release easily assimilated sugars by yeast prior to the use as a substrate. The hydrolysates contain glucose, maltose, oligosaccharides and polysaccharides in a proportion depending on the nature of the utilized enzyme and the degree of hydrolysis. The transformation of whey permeate and starch to feed yeast is an interesting process due to the good nutritional value of yeasts (Lukondeh *et al.*, 2003 and Schultz *et al.*, 2006). However, it has been observed that in aerated cultures of *K. fragilis* and *K. lactis* a change in cellular metabolism from oxidative to a mixed oxidative-fermentative state

can occur. This change causes the production of by-metabolic products such as alcohol, aldehydes, esters, etc., which reduce the yields of biomass from whey (Beausejour *et al.*, 1981, Cristiani-Urbina *et al.*, 1997; Inchaurrondo *et al.*, 1994) To avoid this undesirable effect, the mixed yeast culture has been used Carlotti *et al.*, 1991 and Cristiani-Urbina *et al.*, 2000). It is necessary to note that the major part of the industrial techniques concerning the production of food grade yeast, always rest on a pure culture of a given kind of microorganism.

The objectives of this work was 1) to test several local strains of *Saccharomyces cerevisiae* to choose a strain that has suitable physiological characteristics such as high biomass production, high yield and produced limited quantity of ethanol. 2) To study the influences of the composition of the whey permeate on the growth of the selected *S. cerevisiae* 3) To detect the effect of glucose (starch hydrolysates) on lactose assimilation by *K. marxianus*. 4) To develop a continuous fermentation process to produce cheap proteins implicating two strains of yeast allowed in the food industry, growing on a mixed substrate constituted of whey permeate and starch hydrolysates.

2. MATERIALS AND METHODS

2.1. Micro-organisms

Three strains of *Saccharomyces cerevisiae* (Sc 1, Sc 2, and Sc 3) and one strain of *Kluyveromyces marxianus* were used. The *Kluyveromyces marxianus* was isolated from Jordanian dairy products; however, *Saccharomyces cerevisiae* strains were obtained from brewing industries in Amman area Jordan. The yeasts were maintained slants containing Yeast-Malt media: yeast extract 3 g/l, malt extract 3 g/l, peptone 5 g/l, glucose 10 g/l (Scharlau microbiology)

2.2. Fermentation media

Different types of fermentation media have been used and prepared from Yeast Nitrogen Base or Y.N.B. (Difco) supplemented with different carbon sources: (1)concentrated whey permeate coming from the factory of the University of Mutah south of Jordan. 2) Two different starch hydrolysates A and B were coming from distillery. The sugar composition of hydrolysate A is 97.0 % of glucose, 1.0 % of maltose, 1.5 % of maltotrioses and 0.5 % of polysaccharides. The sugar composition of hydrolysate B is 47.7 % of glucose 43.6 % of maltose, 1.5 of maltotrioses and 6.6 % of polysaccharides. The sterilization of hydrolysates was performed by ultra-filtration at 0.2 µm.

2.3. Experimental design

Subcultures were performed in Erlenmeyer flasks, whereas for the batch and continuous cultures, the experiments were performed in 2 L bioreactor. Each of the following experiments was repeated three times and the averages were calculated.

2.3.1. Batch fermentations

Tables (1 and 2) present the experimental design of the subcultures and of batch cultures, respectively. The subcultures of *K. marxianus* Table 1 (N°2) were conducted on two distinct media, one based on glucose (subculture n°1), the other based on whey permeate (subculture n°2). Each subculture then served for inoculating a fermentor containing fermentation media composed of whey permeate (lactose) and of glucose Table (2).

Cultures of *S. cerevisiae Sc 3* were applied to fermentor containing two different media Table 2 (N°3). The first medium (C°1) constituted of whey permeate associated with glucose as well as Y.N.B in order to make sure of no possible vitamin and amino acid limitation. The second medium (C°2) contains only the whey permeate associate with glucose.

2.3.2. Continuous fermentations

The operational conditions applied to subcultures of *S. cerevisiae Sc 3* were as follows: ethanol (5 g/l), Y.N.B. (6.7 g/l), working volume (100 ml), agitation (250 rpm), temperature (28 °C), pH (4.5) and total incubation time 48 hours. The operational conditions applied to subcultures of *K. marxianus* were as followed: whey permeate (lactose) (5 g/l), yeast extract (0.5 g/l), $FeCl_3$ (2 mg/l), $(NH_4)_2SO_4$ (1.5 g/l), working volume (500 ml), agitation (800rpm), temperature (30°C), pH (4.5) and total incubation time 11 hours .

The plan of the experiment was identical for all the accomplished continuous cultures I, II, III and III-bis. Firstly, a batch culture of *S. cerevisiae Sc 3* on ethanol was performed with the following conditions: ethanol (5 g/l), whey permeate (lactose) (5 g/l), yeast extract (0.5 g/l), $FeCl_3$ (2 mg/l), $(NH_4)_2SO_4$ (1.5g/l), polyethylene glycol (0.5 ml/l), working volume (1.2 l), agitation (800 rpm), aeration (1 vvm), temperature (30°C), pH (4.5). As soon as *S. cerevisiae Sc 3* has assimilated the available ethanol, continuous culture was then started. The following conditions were used for the continuous I, II, III and III-bis:

Table (1): The operational conditions applied to the subcultures of the batch fermentations.

	Subcultures for the batch fermentations			
	N°1	N°2		N°3
		n°1	n°2	
Operation conditions	*S. cerevisiae Sc 1, Sc 2* and *Sc 3*	*K. marxianus*	*K. marxianus*	*S. cerevisiae Sc 3*
Glucose (g/l)	10	10	-	10
Whey permeat (g/l)	-	-	10	-
YNB (g/l)	6.7	6.7		6.7
Y.E. (g/l)	-	-	0.5	-
$FeCl_3$ (mg/l)	-	-	2	-
$(NH_4)_2SO_4$ (g/l)	-	-	1.5	-
Working volume (ml)	100	100	100	100
Agitation (rpm)	250	250	250	250
Temperature (°C)	28	28	28	28
pH	4.5	3.5	3.5	4.5
Duration (h)	14	9	9	14

Yeast Nitrogen Base = Y.N.B., Yeast Extract = Y.E, N°1, N°2 and N°3 = series of experiments for a given microorganism(s)

Table (2): The operational conditions applied to the batch fermentations.

	batch fermentations			
	N°1	N°2	N°3	
			C°1	C°2
Operation conditions	*S. cerevisiae* Sc 1, Sc 2 and Sc 3	*K. marxianus*	*S. cerevisiae* Sc 3	*S. cerevisiae* Sc 3
Glucose (g/l)	10	5	10	10
Whey permeat (g/l)	-	5	10	10
YNB (g/l)	6.7	-	6.7	-
Y.E. (g/l)	-	0.5	-	0.5
$FeCl_3$ (mg/l)	-	2	-	2
$(NH_4)_2SO_4$ (g/l)	-	1.5	-	1.5
P.P.G (ml/l)	-	0.5	0.5	0.5
Working volume (L)	1.2	1.2	1.2	1.2
Agitation (rpm)	600	900	700	700
Temperature (°C)	30	30	30	30
Aeration (vvm)	1	1	1	1
pH	4.5	3.5	4.5	4.5

Yeast Nitrogen Base = Y.N.B., Yeast Extract = Y.E, P.P.G = polyethylene glycol, vvm = volume of air per volume of culture medium per minute, N°1, N°2 and N°3 = series of experiments for a given microorganism(s)

$FeCl_3$ (2 mg/l), $(NH_4)_2SO_4$ (1.5g/l), polyethylene glycol (0.5 ml/l), working volume (1 l), agitation (800 rpm), aeration (1 vvm), temperature (30°C), pH (4.5), dilution rate 0.18 h^{-1}, yeast extract [(0.5 g/l) for continuous I, II and III and (0.1 g/l) for continuous III-bis]. Mixed substrate was constituted of the whey permeate (lactose) (5 g/l) associated either with glucose (5 g/l) for continuous-I or with starch hydrolysate A (5 g/l glucose) for continuous-II and or with starch hydrolysate B (3 g/l glucose and 3 g/l maltose) for continuous-III and III-bis. When a steady state was reached, then 30 ml of a concentrated subculture of *K. marxianus* was added to the fermentor. The method of evaluation of both populations of yeasts is: the method of resistance to the actidione (Van der Walt, 1970) for the continuous-I and the X-Gal method for the continuous-II (Maniatis *et al.*, 1982).

2.4. Analytical methods

All analyses were performed in triplicate and the results were reported as means. Cell-free samples (filtration through 0.2 μ_m Millipore membranes) were used to analyse different

substrates and metabolites of all fermentations. Supernatants were collected and conserved at -18 °C until the time of analysis.

2.4.1. Kinetics parameters determination

At each sampling time, cell density was measured after proper dilution by measuring the optical density at 660 nm (OD_{660}) using a spectrophotometer (Shimadzu model UV-120-02). Biomass concentration was also estimated by pipeting 10 ml samples into pre-weighed dry tubes and centrifuging at 12000 g for 10 min. in refrigerated centrifuge at + 4°C. After removal of the supernatant, cells were washed (twice) by re-suspending in distilled water, centrifuging as previously described. The washed cells were dried in an oven at 104 °C for 24h, after which the tubes were re-weighed and the cell dry weights estimated. The determination of maximum specific growth rates and yields were done from the balance equations on the fermentor.

Enumeration of yeast populations was done using the following:

1) Actidione (cycloheximide) method

The inoculation of Petri dishes was accomplished by inclusion of 1ml of the last dilution of the sample in: 1) 10 ml of Yeast Medium (Y.M.) with agar, 2) 9 ml of Yeast Medium (Scharlau microbiology) with agar, in which 1 ml of a sterile solution of actidione of concentration of 50 mg / l was added. Petri dishes were incubated at 30 °C for 48h. The first series of Petri dishes (Yeast Medium without cycloheximide (Sigma-aldrich) gave the total population of yeast, while the second series (Yeast Medium supplied with actidione) gave the population of *Kluyveromyces*. By difference between both series the population of *Saccharomyces* could be assessed (Van der Walt, 1970).

2) X-gal or 5-bromo-4-chloro-3-indolyl β-D-galactoside method:

The strains of *Saccharomyces* and *Kluyveromyces* were cultivated on Petri dishes, on a medium composed of Yeast Nitrogen Base (Y.N.B) Difco 6.7 g/l, glucose 10 g/l, lactose 10 g/l and X-gal 40 mg/l (Fisher BioReagents). The inoculation was made by streaking of 0.1ml of an appropriate dilution of the sample. Petri dishes were incubated at 30 °C for 48 h. The colonies of *Klyuveromyces* were blue while those of *Sacchromyces* were white (Maniatis *et al.*, 1982).

To determine the sugars in the samples of the yeast culture during fermentation, commercially available enzymatic test kits were used (Boerhinger Mannheim, Germany).

Ethanol and acetic acid determination were measured by gas chromatography, using a DELSI DI-200, with flame ionization detector FID. A column (2 m by 2 mm) was packed with porapak Q 80-100 mesh. The injector and detector temperature were 220 °C and the column oven operated isothermally at 170 °C.

The concentrations of organic acids in the culture broth were determined by using high-performance liquid chromatography (HPLC). A column polypore H. Brownlee was used. The column was eluted at temperature 65 °C with 0.04N H_2SO_4 at a flow rate of 1ml/min. The injection volume was 40 µl with retention time of 10 min. Standards (0.1-0.7 g/l) of pyruvic, formic, citric, malic, and α-ketoglutaric acid (Sigma Chemicals) were filtered through a 0.45 µm filter prior to injection into the column.

To determine the kinetic parameters of growth, biomass production and yields the following equations were used:

The biomass balance equation for batch fermentation can be expressed as follows:

$$r_x''' v = d\frac{(v.x)}{dt} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

r_x''', v and x are biomass formation rate, fermentation volume and biomass concentration, respectively. In batch fermentation the volume, v, is constant then

$$r_x''' v = v.d\frac{(x)}{dt} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(2)$$

If $\mu = \mu_{max}$, then equation (2) becomes:

$$\mu_{max}.x = d\frac{(x)}{dt} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(3)$$

Rearrangement of equation (3) will give:

$$\mu_{max}.dt = d\frac{(x)}{x} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(4)$$

Hence

$$\mu_{max}.t = \ln x - \ln x_0 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(5)$$

$$\ln x = \ln x_0 - \mu_{max} t \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(6)$$

The maximum specific growth rates, μ_{max} (h^{-1}), is therefore, the slope of the curve $LnX = f(t)$. X and X_0 are biomass concentration and initial biomass concentration (g/l), respectively.

The balance equations of glucose, G, in the fermentor will be as follows:

$$-r_G'''V = \frac{d(VG)}{dt}$$ where

$$r_G''' = -\frac{dG}{dt} \quad \text{.....(7)}$$

$$Y_{X/G} = \frac{r_x'''}{r_G'''} = -\frac{dX}{dG} = \frac{X - X^0}{G^0 - G} \quad \text{.....(8)}$$

$$X - X^0 = Y_{X/G^0}(G^0 - G) \quad \text{.....(9)}$$

Where G^0, r_G''' and $Y_{X/G}$ are the initial glucose concentrations (g/l), glucose consumption rates (g/l.h) and the yield of biomass on glucose (g/g), respectively.

The balance equations of ethanol, E (g/l), in the fermentor will be as follows:

First phase: Ethanol production

$$-r_E'''V = \frac{d(VE)}{dt}$$ with

$$r_E''' = \frac{dE}{dt} \quad \text{.....(10)}$$

$$Y_{E/G} = \frac{r_E'''}{r_G'''} = -\frac{dE}{dG} = \frac{E - E^0}{G^0 - G} \quad \text{.....(11)}$$

$$E - E^0 = Y_{E/G^0}(G^0 - G) \quad \text{.....(12)}$$

Where E^0, r_E''' and $Y_{E/G}$ are the initial ethanol concentrations (g/l), ethanol production rate (g/l.h) and the yield of ethanol on glucose (g/g), respectively.

Second phase: Ethanol consumption

$$-r_E'''V = \frac{d(VE)}{dt}$$ with

$$r_E''' = -\frac{dE}{dt} \quad \text{.....(13)}$$

$$Y_{X/E} = \frac{r_X'''}{r_E'''} = -\frac{dX}{dE} = \frac{X - X^{0'}}{E^{0'} - E} \quad \text{.....(14)}$$

$$X - X^{0'} = Y_{X/E^{0'}}(E^{0'} - E) \quad \text{.....(15)}$$

Where $Y_{X/E}$ is the yield of biomass on ethanol (g/g).

When plotting the different curves, was it is possible to determine the yields of biomass on glucose, $Y_{X/G}$, on ethanol, $Y_{X/E}$ and also the yield ethanol on glucose, $Y_{E/G}$.

3. RESULTS AND DISCUSSION

3.1. Batch cultures

3.1.1. Selection of *Saccharomyces cerevisiae* strain

Table (3) summarizes the kinetic parameters resulted from the three tested strains. These strains present the same diauxic growth profile. Presence of glucose concentration was important at the beginning of the fermentation; the yeast growth became rapidly high and attained a growth rate of 0.27 h^{-1} for *S. cerevisiae Sc 2* and 0.32 h^{-1} for *S. cerevisiae Sc 3* (Table 3). Glucose suppressed the respiration of yeast and the accumulation of ethanol was observed. A low biomass yield (0.12 and 0,17 g of dry matter per gram of consumed glucose for *S. cerevisiae Sc 2 and* for *S. cerevisiae Sc 3*, respectively) pointed out a predominant fermentation metabolism. Once glucose was depleted, after few hours of adaptation to the new environment, a second stage of rapid growth took place that corresponds to the use of ethanol.

During this second stage, a growth rate with values of 0.06 and 0.11 h^{-1} for *S. cerevisiae Sc 1* and for *S. cerevisiae Sc 3*, respectively, was obtained and a high yield with values of 0.38 and 0.50 (g of dry matter per gram of consumed glucose) for *S. cerevisiae Sc 2* and for *S.cerevisiae Sc 3*, respectively was obtained. These results are an indicator of ethanol assimilation. The vast majority of yeast strains consumed both maltose and maltotriose only after glucose depletion (Salema-Oom *et al.*, 2005). Moreover, most yeast strains used maltotriose only after maltose was consumed, and very often trisaccharides were not completely consumed (Londesborough, 2001). The obtained results in Table (3) indicate that *S. cerevisiae* Sc 3 showed the highest maximum specific growth rates on glucose, ($\mu_{max\ glu}$) and on ethanol, ($\mu_{max\ eth}$). The biomass yields on glucose ($Y_{X/G}$) and on ethanol ($Y_{X/E}$) were also the most important with the *S. cerevisiae* Sc 3. It had also relatively low yield of ethanol on glucose ($Y_{E/G}$) of a value of 0.33 (g/g). According to the obtained results *S. cerevisiae* Sc 3 was chosen for the next study.

Table (3): The kinetic parameters of *Saccharomyces cerevisiae* strains.

Kinetic parameters	*Saccharomyces cerevisiae*		
	Sc 1	*Sc 2*	*Sc 3*
$\mu_{max\ glu}$ (h^{-1})	0.28	0.27	0.32
$\mu_{max\ eth}$ (h^{-1})	0.06	0.08	0.11
$Y_{X/G}$ (g/g)	0.17	0.12	0.17
$Y_{X/I}$ (g/g)	0.50	0.38	0.50
$Y_{I/G}$ (g/g)	0.44	0.30	0.33

With $\mu_{max\ glu}$: Maximum specific growth rate on glucose, $\mu_{max\ eth}$: Maximum specific growth rate on ethanol, $Y_{X/G}$: yield biomass on glucose, $Y_{X/I}$ yield biomass on ethanol, $Y_{I/G}$: yield ethanol on glucose.

Table (4): Summary of the kinetic parameters, maximum specific growth rates and yields, for the strain of *S. cerevisiae Sc 3* in the cultures media C°1 and C°2 as described in Table 2 (N°3).

Kinetic parameters	Culture media		
	C°1	C°2	Results *
$\mu_{max\ glu}$ (h^{-1})	0.56	0.53	0.32
$\mu_{max\ eth}$ (h^{-1})	0.11	0.10	0.11
$Y_{X/G}$ (g/g)	0.15	0.18	0.17
$Y_{X/I}$ (g/g)	0.44	0.50	0.50
$Y_{I/G}$ (g/g)	0.42	0.37	0.33

$\mu_{max\ glu}$ and $\mu_{max\ eth}$ are the maximum specific growth rates on glucose and on ethanol respectively. $Y_{X/G}$, $Y_{X/I}$ and $Y_{I/G}$ are the the yields biomass on glucose, biomass on ethanol and ethanol on glucose respectively. * For comparison, results come from table 3.

Table (5): The yield, productivity and the percentages of the two flora observed during the steady state of different continuous cultures.

Cultures	Yield $Y_{X/G}$	Productivity	*K. marxianus*	*S. cerevisiae*
	(g/g)	g/l.h	%	%
continuous-I **Permeate + glucose + Y.E 0.5g/l**	**0.47**	**0.8**	**80**	**20**
continuous-II **Permeate + Hydrolysate A + Y.E 0.5g/l**	**0.43**	**0.7**	**100**	**0**
continuous-III **Permeate + Hydrolysate B + Y.E 0.5g/l**	**0.48**	**0.9**	**13**	**87**
continuous-III-bis **Permeate+ Hydrolysate B + Y.E 0.1g/l**	**0.44**	**0.8**	**64**	**36**

3.1.2.Growth kinetics of *Kluyveromyces marxianus* on whey permeate supplied with glucose

The influence of glucose that comes from starch hydrolysates on the assimilation of lactose by *K. marxianus* was studied. In both cases, *K. marxianus* presents an exponential growth up to the exhaustion of substrates (lactose and glucose). Whatever the nature of the carbohydrate source (lactose or glucose) used in the subculture, *K. marxianus* began by using glucose, and then the assimilation of lactose started even if the fermentation medium still contains about 3.5 g/l of glucose. The assimilation of glucose by *K. marxianus* started after 4 hours of incubation in the case where the subculture was realized on lactose, and 1 hour of incubation in the case where the subculture was realized on glucose. The calculation of maximum specific growth rates gives μ_{max} = 0.42 h^{-1} and 0.37 h^{-1} if the subculture was realized on lactose and on glucose, respectively. Therefore, the values obtained for μ_{max} in both cases were nearly similar.

3.1.3. Growth kinetics of *Saccharomyces cerevisiae Sc 3* on whey permeate supplied with starch hydrolysates (glucose)

The influence of the composition of the whey permeate on the growth of *S. cerevisiae Sc 3* was studied to find out if whey permeate could possibly contains inhibitors or be deficient in certain nutrients necessary for *Saccharomyces* growth. The maximal biomass concentration was identical in the two media C°1 and C°2 (Table 2) and it was 3.5 g/l. The biomass reached its maximal concentration after 15 hours of incubation in both media. The total assimilation of glucose was reached after 6 and 7 hours of incubation in the medium C°1 and C°2 (Table 2), respectively. The maximal ethanol production (3.9 g/l and 3.4 g/l) was obtained after 6 and 7 hours of incubation in the medium n°1 and n°2, respectively. The obtained results indicated that there were no significant differences between the growth parameters for the two media C°1 and C°2 Table 2 (N°3). The maximum specific growth rates on glucose and on ethanol ($\mu_{max\ glu}$ and $\mu_{max\ eth}$) for the two media C°1 and C°2 were identical (Table 4). The yields biomass on glucose, $Y_{X/G}$ and biomass on ethanol, $Y_{X/E}$ acquired on medium n°2 are superior to those acquired on medium n°1. Moreover, the yield of ethanol on glucose, $Y_{E/G}$, on medium C°2 was less than that acquired on medium C°1 (Table 4). The addition of Y.N.B to the medium therefore did not improve the performances of the culture. The results of the third column in the Table (4) indicate that medium n°2 allows a better growth (higher in $\mu_{max\ glu}$, $\mu_{max\ eth}$ and excess yield) than the medium Y.N.B supplied only with glucose. As a result, the whey permeate supplied with nutrients necessary for the growth of *K. marxianus* [$FeCl_3$, $(NH_4)_2SO_4$ and Y.E], allowed an optimum growth for *S. cerevisiae*. The obtained results therefore indicated that the whey permeate used in this study did not contain inhibitive elements.

3.2. Continuous cultures

Fig (1 and 2) present the growth kinetics acquired in the fermentation course of the two accomplished studies, continuous-I and continuous-II. At the steady state, there was no accumulation of sugar in the medium (lactose or glucose), and ethanol production was equal to zero. The yields of yeast on sugar ($Y_{X/G}$) calculated from the final quantity of produced yeast, to the total quantity of consumed sugars (lactose and glucose), were equal to 0.47g/g and 0.43g/g for continuous-I and continuous-II, respectively (Table 5). The productivity (calculated as final quantity of produced yeast multiplied by the dilution rate) was equal to 0.8 and 0.7 g/l.h for continuous-I and continuous-II, respectively. It was found that in continuous-I, at the steady state, *K. marxianus* was represented 80 % of the total population while *S. cerevisiae Sc 3* was represented only 20 % (Table 5). In the continuous-II, the population of *S. cerevisiae Sc 3* has completely exceeded by that of *K. marxianus*. The obtained percentages for the continuous-I were indeed inexact seen to the used methodology for the evaluation of the two yeasts (method of resistance to actidione). Because the evaluation of the population of *S. cerevisiae Sc 3* being accomplished by difference of the obtained populations on the medium with and without actidione, the difference between the two method was almost inexistent. The determined percentages by the X-Gal method in the case of the continuous-II were more reliable and consequently, they show that it was not possible to support in a stable way both strains in co- culture in the conditions of the continuous-I and continuous-II. The displacement of the population of *S. cerevisiae Sc 3* by the growth of *K.*

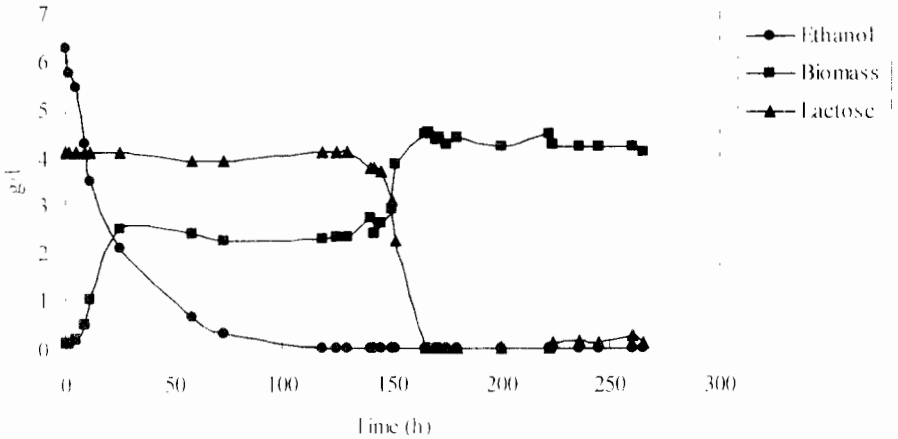

Fig. (1): Continuous culture I on whey permeate (lactose 5 g/l) supplied with glucose 5 g/l

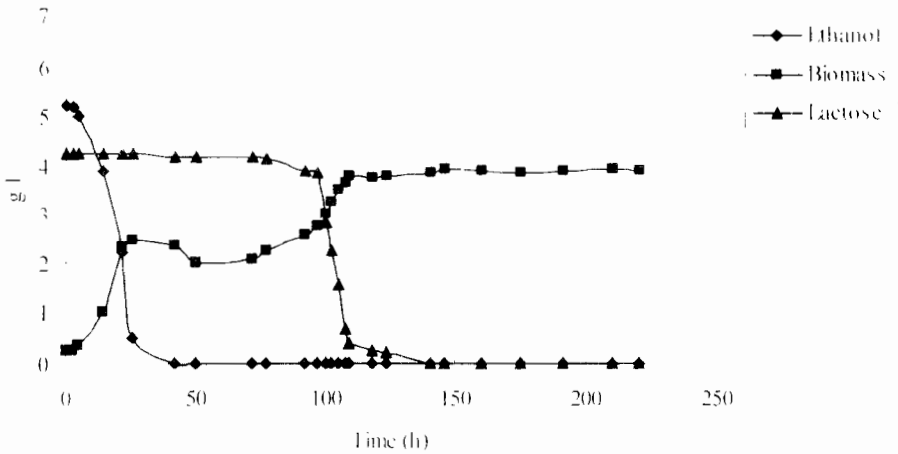

Fig. (2): Continuous culture II on whey permeate (lactose 5 g/l) supplied with starch hydrolysate 'A' (glucose 5 g/l).

marxianus seems to be explained by the consumption of glucose of the medium, which was faster in the *Kluyveromyces* cells. Gasnier (1987) showed that in lactose-grown cells, glucose was transported in *K. marxianus* through a high affinity H^+-sugar symporter (Km=0.09mM), whereas a low-affinity transporter (Km=3.5 mm) was utilized in glucose-grown cells. Compared with the values of the Km of transporters of glucose in *S. cerevisiae* cells (Km = 1-5 mm and Km = 20-50 mm), the capture of glucose from the medium is therefore accomplished with a higher affinity in the *Kluyveromyces* cells than in *Saccharomyces* cells. The impossibility of stabilizing a coculture on a mixed substrate containing whey permeate and glucose was the reason to test the effect of other carbohydrate sources than glucose. Starch hydrolysate "B", with high maltose content, was then tested with the whey permeate as a new mixed substrate. It is important to know that the maltose is assimilated only by *Saccharomyces* cells. The following fermentations of continuous-III and continuous-III-bis were performed for studying the behavior of the coculture in the presence of the new mixed substrate. Figure (3) presents the obtained kinetics in the course of continuous cultures. It was observed that there were no production of ethanol and accumulation of glucose in the culture medium. However, residual maltose in a constant concentration (0.2 g/l) was found. A slight accumulation of lactose at the rate of 0.1 g/l was also detected when the concentration of yeast extract in the feeding medium was reduced from 0.5 to 0.1 g/l. The yield of yeast on sugar ($Y_{X/S}$) and the productivity for the continuous-III were equal to 0.48 g/g and 0.9 g/l.h, respectively. In the case of continuous-III-bis the yield ($Y_{X/S}$) and the productivity were equal to 0.44 g/g and 0.8 g/l.h, respectively. The obtained results from continuous-III concerning the evaluation of the percentages of the two yeast strains by using the method X-Gal, indicated that 87 % of the population belonged to the *S. cerevisiae Sc 3* and 13 % of the population belonged to *K. marxianus* (Table 5). These results were unexpected.

If it is assumed that a theoretical yield (Y_t) is equal to 0.5 g/g (gram of *K. marxianus* produced per gram of consumed lactose), and knowing that, the total biomass (X) becomes stable to a value of about 5 g/l and there is no lactose residues in the medium (Fig. 3), therefore, the percentage of *K. marxianus* cells should at least be equal to $\frac{Y.S_0}{X}100 = 50\%$, where S_0 is the initial lactose concentration in the feeding and is equal to 5 g/l. This percentage can of course be higher if taking into account the partial or total assimilation of the available glucose in the medium by *K. marxianus* as in the case of continuous-I and continuous-II.

The obtained experimental percentages from continuous-III-bis were 36% for the *S. cerevisiae Sc 3* and 64 % for *K. marxianus*. These results are in agreement with the theoretical percentages if it is suppose that:
(Y) is the yield of conversion of each of the sugars into biomass and it was assumed to be equal to 0.5 g/g.
(X) is the total biomass at the steady state which was experimentally measured as equal to 4.5 g/l (Fig. 3), (S^0) is the initial sugars concentration in the feeding. S is the concentration of residual sugars in the medium

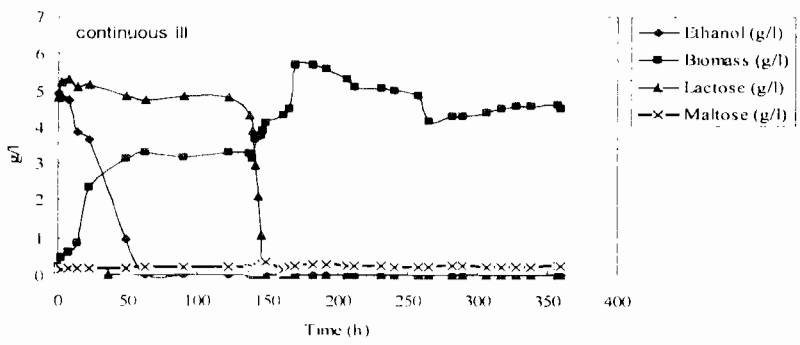

Fig. (3): Continuous culture III and III-bis on whey permeate (lactose 5 g/l) supplied with starch .

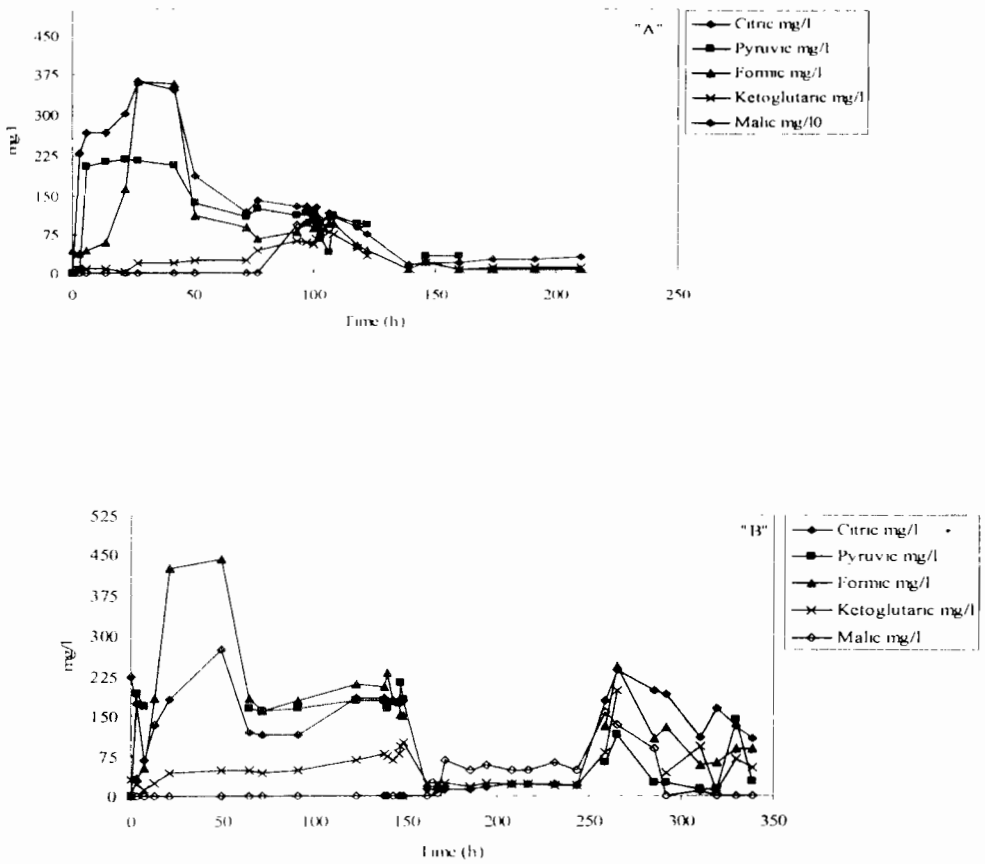

Fig. (4): Production of organic acids during "A" Continuous II and "B" during continuous III and III-bis. Y.E=Yeast extract.

it is experimentally measured and it was equal to 0.1 g/l for lactose and 0.2 g/l for the maltose (Fig. 3). If it is assumed that *S. cerevisiae Sc 3* only assimilates maltose while *K. marxianus* only assimilates lactose, the following theoretical percentages should be obtained:

$$\%_{th}\ \text{S.c} = \frac{Y(S_o - S)}{X}100 = \frac{0.5(3-0.2)}{4.5}100 = 31.11\%.$$

$$\%_{th}\ \text{K.m} = \frac{Y(S_o - S)}{X}100 = \frac{0.5(5-0.1)}{4.5}100 = 54.44\%.$$

These theoretical percentages are slightly less than the experimental percentages, which are wholly logical since they do not take into account the production of yeast from the consumption of glucose. According to the study of Busturia and Lagunas (1985), the system of transport of the maltose in the *Saccharomyces* exists in two forms with Km equal to 4 and 70 mM, respectively.

It was concluded that both forms of the maltose transport system were regulated by catabolite inactivation. Therefore, this system by the values of Km and by the mechanism of regulation looks like the system of transport of glucose in the same yeast. It would remain to define the influence of the maltose on the metabolism of lactose by *K. marxianus* since the results of the continuous-III gave in the steady state, 13 % *K. marxianus* and to be compared with 100 % acquired in the case of the continuous-II. On the other hand, when the concentration in yeast extract of the medium was reduced from 0.5 to 0.1 g/l, the metabolism of lactose by *K. marxianus* was once again performed in an usual manner: because 64 % of the cells were produced, which is in agreement with the theoretical percentage.

3.3. Analysis of the evolution of the organic acids concentration

The evolution of the concentration of several organic acids was followed during the course of the continuous fermentations II (Fig. 4A), continuous fermentations III and III-bis (Fig. 4B). These organic acids are Pyruvic acid, Formic acid, Citric acid, Malic acid, α-Ketoglutaric acid. In the course of the batch fermentation of *S. cerevisiae* on ethanol, there was a massive production of the above mentioned organic acids. This production was always present but tends to become stable in the course of the continuous cultures. The addition of *K. marxianus* to the medium led to an important fall in the concentration of all acids, there was undoubtedly a consumption of these acids by such yeast. In the presence of maltose (continuous-III), this consumption could possibly cause an energy uncoupling at the cell level, and led to a poor conversion of lactose into biomass, which would explain the low percentage (13 % of cells) of produced *K. marxianus*. When the concentration of yeast extracts in the feeding was reduced to 0.1 g/l, the assimilation of the organic acids by *K. marxianus* was distinctly less important than in the case where yeast extract concentration was equal to 0.5 g/l. At a concentration of 0.1 g/l, the yeast extract, therefore, prevented the assimilation of acids by *K. marxianus*, and it followed that the metabolism of lactose was once again accomplished in an usual way, leading to a resumption of cells viability.

This study reveals that the strain *S. cerevisiae* Sc 3 was the most suitable among the three tested strains which has the most efficient cellular multiplication process. The importance of this work resides also in the transformation of two different agro-industrial wastes (whey permeate and starch hydrolysates) by two different yeast strains (*S. cerevisiae Sc 3* and *K. marxianus)* in a stabilized coculture. Also, this kinetic study has presented the advantages of this coculture: the diversity and richness of the obtained biomass and the joint utilization of two agro-industrial wastes. The food industries will benefit by having available an alternative to the disposal of their wastes. The transformations of whey permeate and starch hydrolysates to a viable product such as single cell protein offers an effective solution to the environmental disposal problem of the two wastes.

Acknowledgements

I would like to thank Dr. Malik Haddadin from Jordan University, Amman-Jordan and Dr Catherine Valance from Bongrain Gerard SA, Le Tholy-France for skilled technical and analytical assistance.

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أنتاج مادة حيوية بروتينية من مخلفات الصناعات الغذائية (الشرش والنشا) عن طريق استخدام ثلاث سلالات من خميرة *Saccharomyces cerevisiae* وسلالة واحدة من خميرة *Kluyveromyces marxianus*

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ملخص

يعتبر كل من المصل (الشرش) والنشا من مخلفات الصناعات الغذائية التي امكن تحويلها عن طريق تغذية الخمائر عليها لإنتاج مادة حيوية بروتينية. وترجع اهمية هذه الدراسة الى القيمة الغذائية العالية للخمائر كغذاء للإنسان والحيوان هذا فضلا عن تخليص البيئه من هذه المخلفات. أستخدم في هذة الدراسة ثلاث سلالات من خميرة *Saccharomyces cerevisiae* هي (*Sc 1*, *Sc 2* and *Sc 3*) وسلالة واحدة من خميرة *Kluyveromyces marxianus*. أعطت السلالة (*S. cerevisiae Sc 3*) أعلى معدل نمو بوجود الجلوكوز ($0.32\ h^{-1}$) و بوجود الأيتانول ($0.11h^{-1}$) تحت ظروف مزارع الدفعات بالاضافة ألى أعلى محصول للخلايا ولهذا استخدمت في مزرعة مختلطة مع *Kluyveromyces marxianus*. في الجزء الثاني من الدراسة والخاص بانتاج المادة الحية من هذه الخلايا من المخلفات بالطريقة المستمرة. وقد اثبتت الدراسة أمكانية السيطرة على عملية الانتاج للمادة الحية على المستوى الصناعي وذلك بالوصول الى ثبات ديناميكية النمو باستخدام المزارع المختلطة مع مخلفات الصناعات الغذائية.

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