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Influence of substrate on β-galactosidase production by *Kluyveromyces* strains

Manuela ROLLINI*, Valentina TRINETTA, Alida MUSATTI, Matilde MANZONI

DiSTAM, Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Sezione di Microbiologia Industriale, Università degli Studi, Via G. Celoria 2, 20133 Milano, Italy

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Abstract - The aim of the present research was to investigate the influence of culture conditions on the levels of β-galactosidase (EC 3.2.1.23) activity produced by *Kluyveromyces* strains. Interest was focused on evaluating enzyme activity levels when lactose or cheese whey was employed as substrate in culture medium formulation. From an overall look at the obtained results, the tested strains were found to be able to produce β-galactosidase at promising levels. The use of cheese whey, either for strain maintenance and production trials, allowed to obtain a high cell yield associated with β-galactosidase production. The maximum β-galactosidase volumetric activity, EA_{max} 66.5 IU/ml, corresponding to 3184 EA_{spec} IU/g cell dw, was obtained with *K. marxianus* MIM 782 at 37 °C and 72 h incubation.

Key words: lactase, β-galactosidase, cheese whey, Kluyveromyces, lactose hydrolysis.

INTRODUCTION

Lactose, the main carbohydrate present in milk, is a disaccharide with relative low sweetness and solubility, not digested by a significant fraction of the population. Not metabolized by β-galactosidase deficient subjects, it arrives un-hydrolyzed in the large intestine where its osmotic effect can lead to tissue dehydration and fermentation of this sugar by the intestinal microbiota, which produces gases causing fermentative diarrhoea, bloating, flatulence and cramps (Panesar *et al.,* 2006). Moreover from the technological point of view, lactose causes many disadvantages in refrigerated foods, such as crystallization in dairy products, development of sandy texture and deposit formation (Mahoney, 1997).

The contingency disposal of lactose present in cheese whey, a by-product of dairy industry, is associated with high values of biological and chemical oxygen demand (BOD and COD), which may produce serious pollution problems to the environment. Its technological valorisation may represent a valid alternative to waste treatment (Ghaly and Kamal, 2004; Panesar *et al.,* 2006; John *et al.,* 2007). Lactose hydrolysis provides several advantages from different points of view, such as nutritional and technological; moreover glucose and galactose are sweeter and more soluble than lactose. The economic interest in β-galactosidase production is related to food and pharmaceutical large scale applications (Furlan *et al.,* 2000; Fonseca *et al.,* 2008).

β-Galactosidase (E.C. 3.2.1.23), commonly known as lactase, can be obtained by a wide variety of natural sources, such as microorganisms, plants and animals, with a marked difference in their properties. Among these possibilities, microbial sources offer several advantages, such as easy handling and high production yields. β-Galactosidase commercially available is obtained from microorganisms of different genera (Panesar *et al.,* 2006), in particular from yeasts belonging to the genera *Kluyveromyces* and *Candida*, from the fungus *Aspergillus*, and from *Bacillus* spp. and *E. coli* (Thigiel and Deak, 1989; Pinheiro *et al.,* 2003).

In large scale β-galactosidase production process, enzyme recovery costs lie at the primary level of production and purification. One of the approaches to limit the production costs is based on finding, with traditional or innovative procedures, strains with high enzyme productivity (Belem and Lee, 1998; Panesar *et al.,* 2006).

The aim of the present research was to set-up a culture medium formulation, suitable for large scale production, by evaluating the influence of substrate on β-galactosidase volumetric and specific productivities. A screening among strains belonging to the *Kluyveromyces* genus was carried out, to find out those suitable for scale-up procedure. *Kluyveromyces* yeast strains offer great advantages, such as a good growth yield, acceptability as safe microorganisms and β-galactosidase activity higher than other yeast strains. Particular interest was focused on evaluating β-galactosidase production levels by replacing lactose with cheese whey.

* Corresponding author. Phone: +39-02-50319150;
Fax: +39-02503119238; E-mail: manuela.rollini@unimi.it

MATERIALS AND METHODS

Microorganisms and culture conditions. Screening for β-galactosidase production was carried out employing strains belonging to the *Kluyveromyces* genus. In particular nine *K. marxianus* strains were obtained from the Centraalbureau voor Schimmelculturen international collection (CBS 834, 397, 607, 1553, 1970, 2231, 2235, 2762, 4857). Two strains were previously isolated and identified as *K. marxianus* at the University of Milan (MIM 782 and 271), as well as one *K. lactis* strain (MIM 96).

Cultures were monthly maintained on different solid media, Malt Extract agar (MEA) and TN-Lactose agar (TN-LA), having the following composition (g/l):
- MEA: malt extract (Costantino, Favria, Italy) 20, soybean peptone (Costantino) 1, glucose 20, agar 15, pH 5.8;
- TN-LA: $(NH_4)SO_4$ 5, K_2HPO_4 1, $MgSO_4$ 0.2, yeast extract (Costantino) 1, lactose 10, agar 15, pH 5.5.

For β-galactosidase production, liquid TN-Lactose (TN-L) (TN-LA composition without agar), and M-W media were employed, the last having the following composition (g/l): cheese whey 70, yeast extract (Costantino) 10, pH 5.8. Media were all sterilised at 118 °C for 20 min. Cheese whey samples were supplied from Sigma® (W1500, Whey1) and Mucedola® (Mucedola srl, Settimo Milanese, Italy) (28015A, Whey2).

β-galactosidase production. Fermentation trials were carried out in 1 l Erlenmeyer flasks, containing 100 ml of medium, inoculated (10% v/v) with a 48 h-old liquid pre-culture, obtained by inoculating 5 ml cell suspension (10^9 cells/ml) from a 48-72 h-old solid culture. Cultures were incubated on a rotary shaker (200 rpm) at different temperatures. Biomass growth was determined as dry weight.

Enzyme assay. Enzyme determination was carried out on treated cells (Lee *et al.,* 2004). Culture samples (3 ml) were centrifuged at 9000 rpm for 5 min and separated cells were washed twice with 0.03 M phosphate buffer pH 6.8 and then suspended in 3 ml of 50% v/v ethanol. After stirring for 15 min at 4 °C, treated cells were separated by centrifugation at 5000 rpm for 5 min. Obtained cells were resuspended in 1 ml phosphate buffer and subjected to β-galactosidase determination.

β-Galactosidase activity was determined according the procedure described by Wallenfels (1962). Briefly, 1 ml of cell suspension was added with 4 ml of 0.05% v/v ONPG (2-nitrophenyl-β-D-galactopyranoside) in 0.03 M phosphate buffer pH 6.8. Samples were then incubated at 40 °C for 15 min. Reaction was stopped by boiling samples for 10 min, which were subsequently cooled in ice. Samples were then centrifuged and *o*-nitrophenol (ONP) concentration was determined spectrophotometrically on the obtained supernatants (OD 405 nm). One unit of β-galactosidase was expressed as the amount of enzyme that hydrolyses 1 μg of ONP in 1 min, in the assay conditions.

RESULTS

Preliminary screening

A screening was carried out among yeasts, belonging to the genus *Kluyveromyces,* in order to evidence high β-galactosidase producer strains. In this phase the influence of MEA (malt extract agar) and TN-LA (TN-Lactose Agar) media employed to maintain the yeast strains, on β-galactosidase production, was evaluated. Production was carried out in TN-L liquid medium, containing lactose acting as carbon and energy source as well as enzyme inducer.

Being the β-galactosidase present on cell surface in cryptic form, cells have to be treated before enzyme determination. Different procedures are reported in literature to achieve the contact between β-galactosidase with substrate (Decleire *et al.,* 1987, Flores *et al.,* 1994, Kippert, 1995), but in our experiments better results were obtained by treated cells with ethanol (Lee *et al.,* 2004). The set up of cell treatment was carried out by evaluating the experimental conditions, in particular time effect (from 5 min to 4 h), temperature (from 5 to 20 °C) and ethanol concentration (from 5 to 60% v/v). Highest β-galactosidase levels were evidenced by treating cells (washed twice with 0.03 M phosphate buffer pH 6.8) at 4 °C for 15 min employing an ethanol concentration of 50% v/v (data not shown). Enzyme levels were comparatively determined on untreated cells (control sample). Obtained data were statistically treated (two-sample *t* test with different variance) to evaluate significance.

From this preliminary research twelve strains were selected. Low biomass yields were evidenced for all samples, without sig-

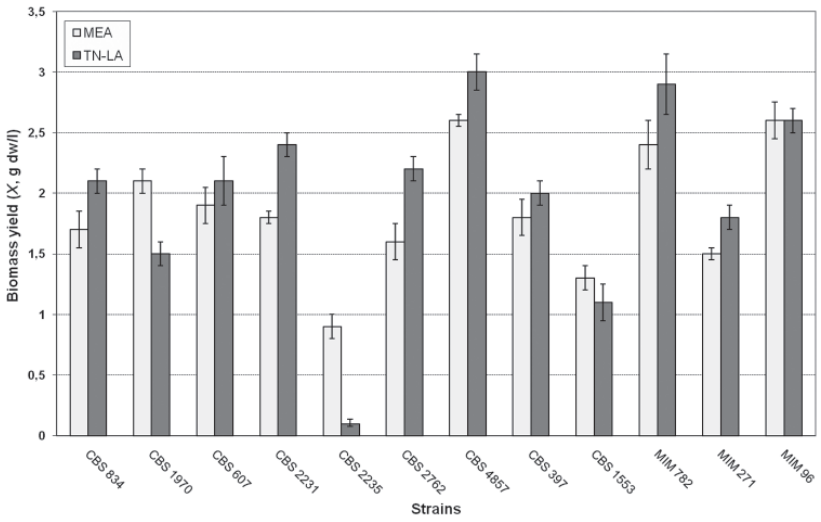

FIG. 1 - Biomass yields (X, g dw/l) of *Kluyveromyces* strains grown in TN-L liquid medium at 28 °C for 48 h. The cells were previously comparatively maintained on MEA and TN-LA media.

TABLE 1 - Effect on β-galactosidase activity of ethanol treatment on cells of *Kluyveromyces* strains comparatively maintained on MEA and TN-LA media before growth in TN-L liquid medium at 28 °C for 48 h

Strain	Cell treatment	EA_{spec} (IU/g dw)*		EA_{max} (IU/ml)**	
		MEA	TN-LA	MEA	TN-LA
CBS 834	Control	150 ± 14	909 ± 81	0.3 ± 0.02	1.4 ± 0.1
	ETOH	344 ± 21	1516 ± 122	0.6 ± 0.04	3.2 ± 0.3
CBS 1970	Control	67 ± 20	736 ± 62	0.1 ± 0.01	1.1 ± 0.1
	ETOH	1181 ± 126	6353 ± 523	2.5 ± 0.3	9.5 ± 0.8
CBS 607	Control	1687 ± 180	3207 ± 300	3.2 ± 0.3	6.7 ± 0.5
	ETOH	3454 ± 29	3831 ± 264	6.6 ± 0.7	8.0 ± 0.6
CBS 2231	Control	2298 ± 159[a]	1710 ± 125	4.1 ± 0.4	4.1 ± 0.3
	ETOH	2382 ± 241[a]	3628 ± 251	4.3 ± 0.4	8.7 ± 0.8
CBS 2235	Control	0	0	0	0
	ETOH	0	0	0	0
CBS 2762	Control	0	379 ± 22	0	0.8 ± 0.07
	ETOH	0	630 ± 59	0	1.4 ± 0.1
CBS 4857	Control	1624 ± 172[a]	2133 ± 133	4.2 ± 0.4[a]	6.4 ± 0.6
	ETOH	1707 ± 116[a]	1787 ± 10	4.4 ± 0.5[a]	5.4 ± 0.5
CBS 397	Control	1552 ± 135	1895 ± 155	2.8 ± 0.2	3.8 ± 0.3
	ETOH	2274 ± 210	2350 ± 214	4.1 ± 0.4	4.7 ± 0.5
CBS 1553	Control	542 ± 61	1900 ± 166	0.7 ± 0.06	2.1 ± 0.1
	ETOH	4148 ± 401	6969 ± 452	5.4 ± 0.5	7.7 ± 0.6
MIM 782	Control	0	1356 ± 147	0	3.9 ± 0.4
	ETOH	1615 ± 144	3118 ± 308	3.9 ± 0.3	9.0 ± 1.0
MIM 271	Control	0	1336 ± 114	0	2.4 ± 0.3
	ETOH	0	1654 ± 187	0	3.0 ± 0.2
MIM 96	Control	683 ± 57	1341 ± 157	1.8 ± 0.2	3.5 ± 0.4
	ETOH	2524 ± 298	3694 ± 365	6.6 ± 0.6	9.6 ± 0.9

* EA_{spec}: specific activity, ** EA_{max}: volumetric activity.
Data are presented as mean ± standard deviation; [a]: means without significant difference ($P < 0.05$).

nificant differences attributable to the solid medium employed for strains maintenance (MEA and TN-LA) (Fig. 1). Table 1 reports β-galactosidase production, expressed as volumetric (EA_{max}, IU/ml) and specific activity, the latter referred to cell dry weight (EA_{spec}, IU/g dw). Cells treated with ethanol generally furnished higher β-galactosidase levels with respect to untreated cells. The highest volumetric activity (8.0-9.6 IU/ml) was evidenced in cells maintained on TN-LA, for *K. marxianus* CBS 1970, 607, 2231, MIM 782 and *K. lactis* MIM 96. Lower levels (up to 6.6 IU/ml) were found in cells maintained on MEA, even if the biomass yield reached similar values. The highest β-galactosidase specific activity was evidenced for *K. marxianus* CBS 1553 strain, with a maximum of about 7000 IU/g dw, associated to a moderate growth (about 1.1 g dw/l), by maintaining the strain on TN-LA medium, while the activity was 4148 IU/g dw (about 38% lower) (1.1 g dw/l biomass yield) on MEA medium.

The obtained results pointed out that cells grown on lactose as carbon and energy source were found to produce interesting β-galactosidase activity at laboratory level; nevertheless the only moderate biomass yield reached (max 3 g dw/l) represents the major limitation for process scale-up.

Cheese whey as substrate

From among the twelve screened strains, only four (*K. marxianus* CBS 1970, 2231, MIM 782 and *K. lactis* MIM 96) were selected for the second part of research, aimed at improving the process. Strains were grown on M-W liquid medium, composed by yeast extract and cheese whey. This set of experiments was performed by substituting lactose with cheese whey, a cheap complex ingredient, suitable for industrial application. Two types of commercial cheese whey were tested, one in a purified form (Whey1) and the other in a raw form (Whey2). Experiments were comparatively carried out at 28 and 37 °C, conventional temperatures used in most microbiological large scale process (Rech *et al.,* 1999; Rajoka *et al.,* 2003).

When employing Whey1, the purified form, and MW medium, biomass yields increased up to 16-22 g dw/l, nearly 8-10 times higher than what previously evidenced employing lactose as substrate (TN-L medium, 0.1-3.0 g cells dw/l), without significant influence of incubation temperature, except for *K. marxianus* MIM 782 strain, that showed a negligible growth at 37 °C (Fig. 2A). As regards β-galactosidase activity levels, only *K. marxianus* CBS

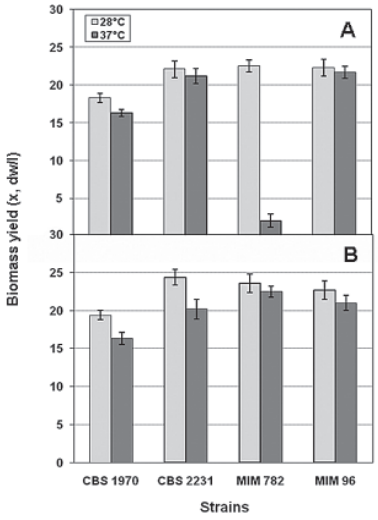

FIG. 2 - Biomass yields (X, g dw/l) of four *Kluyveromyces* strains grown in MW liquid medium containing purified cheese whey (Whey1, A) or raw cheese whey (Whey2, B) at 28 and 37 °C for 48 h. The cells were previously maintained on TN-LA medium).

TABLE 2 - Effect on β-galactosidase activity of ethanol treatment on cells of *Kluyveromyces* strains maintained on TN-LA medium before growth in MW liquid medium containing purified cheese whey (Whey1), for 48 h, at 28 and 37 °C

Strain	Cell treatment	EA_{spec} (IU/g dw)		EA_{max} (IU/ml)	
		28 °C	37 °C	28 °C	37 °C
CBS 1970	Control*	173 ± 12	86 ± 5	3.2 ± 0.3	1.4 ± 0.2
	ETOH	260 ± 15	260 ± 19	4.8 ± 0.5	4.2 ± 0.4
CBS 2231	Control	801 ± 83	888 ± 65	17.7 ± 1.5	18.8 ± 1.6
	ETOH	1072 ± 94	1007 ± 97	23.7 ± 1.9	21.4 ± 1.7
MIM 782	Control	942 ± 86	660 ± 42	21.2 ± 2.6	13.5 ± 1.4
	ETOH	1083 ± 99	931 ± 68	24.4 ± 1.9	19.1 ± 2.0
MIM 96	Control	552 ± 43	466 ± 51	12.3 ± 1.4	10.1 ± 1.1
	ETOH	844 ± 78	910 ± 85	18.8 ± 1.8	19.7 ± 2.2

* EA_{spec}: specific activity, ** EA_{max}: volumetric activity.
Data are presented as mean ± standard deviation. Data obtained from different cell treatment are all significantly different ($P < 0.05$, two-sample *t* test).

1970 showed volumetric and specific activities lower (4.2-4.8 IU/ml and 260 IU/g, respectively) than those previously obtained employing lactose (9.5 IU/ml and 6353 IU/g respectively) (Table 2). The other three strains evidenced at 28 °C volumetric activities of about 18.8-24.4 IU/ml, nearly twice the values previously obtained. β-Galactosidase specific activities instead showed lower levels (840-1100 IU/g), as a consequence of higher biomass yield; the best results were obtained with *K. marxianus* MIM 782 at 28 °C (1083 IU/g dw and 24.43 IU/ml). To be noted that, as evidenced before, treated cells showed highest β-galactosidase activity.

Employing Whey2, the raw ingredient form, biomass yields resulted in the range 16-24 g dw/l, similar to those obtained with Whey1 (Fig. 2B). β-galactosidase activity resulted higher in three up to four yeast strains (Table 3). Again employing *K. marxianus* CBS 1970 β-galactosidase volumetric and specific activities (4.1-4.9 IU/ml and 250 IU/g, respectively) were found lower than those evidenced with lactose as substrate. The other strains gave, lower specific and higher volumetric activities with respect to values obtained employing lactose. The best result was achieved with *K. marxianus* CBS 2231, by incubating the cultures at 37 °C (2166 IU/g dw, 43.7 IU/ml).

From the overall obtained results it can be highlighted that the use of whey, in medium formulation, produced an increase of biomass yields nearly 8-10 times with respect to those obtained with lactose, and consequently high β-galactosidase volumetric productivity, representing the enzymatic activity effectively present per unit (ml) of culture medium.

As regards the two whey form comparatively tested, the raw ingredient allowed to achieve highest biomass yield and β-galactosidase activity levels. In particular, with raw whey higher specific activity levels (up to 2166 IU/g dw) were reached than proved with the purified one (up to 1083 IU/g dw). Volumetric productivity resulted high for at least three up to four strains (25.6-43.7 IU/ml). To be noted that employing the raw cheese whey, cultures incubation at 37 °C allowed to obtained higher β-galactosidase activity.

From the obtained results it can be concluded that the cheaper raw whey can be employed with success in applicative processes for β-galactosidase production.

β-Galactosidase time course production

Experiments were then performed employing *K. marxianus* CBS 2231, MIM 782 and *K. lactis* MIM 96 and the MW liquid medium supplemented with raw whey. The same medium was supplemented with agar (M-WA) and employed also for cell maintenance. Trials were carried out by incubating cultures at 28 and 37 °C, monitoring β-galactosidase production up to 72 h. In this set of trials β-galactosidase activity was determined only on ethanol treated cells.

Results pointed out that volumetric activity and biomass yields always reached the highest values at 72 h incubation time, but with a different behaviour with respect to the temperature. Volumetric activity resulted higher, for all the samples, at 37 °C, and not temperature-related.

TABLE 3 - Effect on β-galactosidase activity of ethanol treatment on cells of *Kluyveromyces* strains maintained on TN-LA medium before growth in MW liquid medium containing raw cheese whey (Whey2), for 48 h, at 28 and 37 °C

Strain	Cell treatment	EA_{spec} (IU/g dw)		EA_{max} (IU/ml)	
		28 °C	37 °C	28 °C	37 °C
CBS 1970	Control	130 ± 11	19 ± 5	2.5 ± 0.2	0.3 ± 0.02
	ETOH	254 ± 32	249 ± 30	4.9 ± 0.5	4.1 ± 0.4
CBS 2231	Control	684 ± 54	715 ± 66	16.7 ± 1.2	14.4 ± 1.4
	ETOH	1356 ± 98	2166 ± 195	33.1 ± 3.3	43.7 ± 3.6
MIM 782	Control	444 ± 57	195 ± 21	10.5 ± 1.0	4.4 ± 0.4
	ETOH	108 ± 11	1669 ± 154	25.6 ± 2.0	37.6 ± 3.7
MIM 96	Control	541 ± 55	271 ± 32	12.3 ± 1.0	5.7 ± 0.5
	ETOH	1299 ± 142	1765 ± 163	29.5 ± 3.7	37.1 ± 4.0

* EA_{spec}: specific activity, ** EA_{max}: volumetric activity.
Data are presented as mean ± standard deviation. Data obtained from different cell treatment are all significantly different ($P < 0.05$, two-sample *t* test).

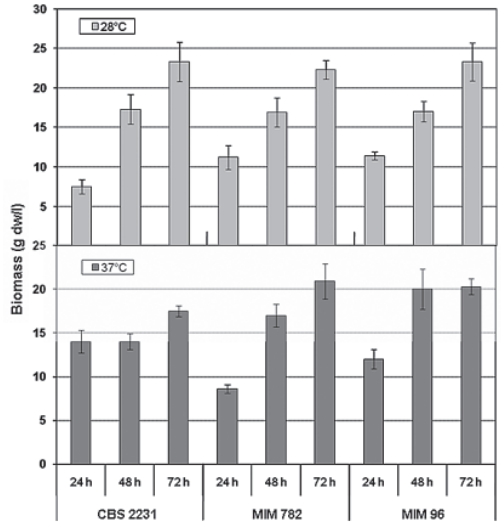

FIG. 3 - Biomass yields (X, g dw/l) of three *Kluyveromyces* strains grown in MW liquid medium containing raw cheese whey (Whey2) at 28 and 37 °C. The cells were previously maintained on solid MW medium.

Kluyveromyces marxianus CBS 2231 reached the highest biomass yield at 72 h incubation, being 23.3 g dw/l at 28 °C, and 17.5 g dw/l at 37 °C (Fig. 3). The highest β-galactosidase volumetric activity was present in cells obtained at 37 °C after 72 h of incubation, with a maximum value of 58.9 IU/ml. Specific activity at 28 °C was higher at 24 h incubation time, reaching 4851 IU/g, to decrease for longer incubation time, while at 37 °C the maximum value, 3543 IU/l, was reached at 48 h (Table 4).

Also for the strains *K. marxianus* MIM 782 and *K. lactis* MIM 69 at 72 h cell yield reached the highest value (about 20-23 g dw/l), independently from the incubation temperature. As regards β-galactosidase levels, a similar behaviour was observed with respect to the previous strain: highest specific activity at 24 h (about 2300- 4300 IU/g dw), while volumetric activity was at maximum level (about 50-67 IU/ml) at 72 h, a longer incubation time.

From an overall look at the obtained results, it can be highlighted that β-galactosidase productivity levels were found significantly higher (up to 66.5 IU/ml) with respect with those obtained in previous experiments. The combination 37 °C-longer incubation time (72 h) allowed to obtain the best results with all the three tested strains (3368, 3184 and 2956 IU/g and 58.9, 66.5 and 60.1 IU/ml respectively for MIM 2231, 782 and 69 strains). To be noted that at 72 h and 37 °C, with the exception of CBS 2231, biomass yields resulted higher than 20 g dw/l.

DISCUSSION

β-Galactosidase production by strains belonging to the genus *Kluyveromyces*, using low cost cheese whey as ingredient in culture medium formulation, proved suitable for applicative purposes in industrial processes. Cheese whey, a polluting by-product of the dairy industry, generally represents a very interesting ingredient for microbial cultures. From an overall look at the results, the tested strains were found to be able to produce β-galactosidase at good levels. In particular between the two whey forms tested in the present research, the low-cost and less purified one allowed to achieve the best results. The maximum β-galactosidase volumetric activity, 66.5 IU/ml, related to 3184 IU/g dw productivity, obtained by *K. marxianus* MIM 782 at 37 °C after 72 h incubation time, represents an interesting starting point for the set up, and at the same time process scale up, on industrial scale, with respect to literature data.

Ornelas *et al.,* (2007), reported a maximum lactase activity of 341.18 mmol ONP min^{-1} g^{-1} (i.e. IU/g) employing a continuous culture of *K. lactis* grown at 30 °C on cheese permeate, near 10-fold lower than the presented data. Furlan *et al.* (2000) reached 34 U/ml growing *K. marxianus* strain in a lactose-free medium, containing molasses and corn steep liquor. Rech *et al.* (1999) obtained 10 U/ml growing *K. marxianus* CBS 712 in a 2-l aerated bioreactor employing 210 g/l cheese whey, 37 °C, 500 rpm and 3 vvm air inlet. Pinheiro *et al.* (2003) reported a significant increase from 5.8 to 17.0 U/g dw using a 6-bar air pressure instead of air at atmospheric pressure with a *K. marxianus* strain. Cortes *et al.* (2005) reached 31700 IU/l and 2800 IU/g dw employing always a *K. marxianus* strain in an aerated bioreactor, manipulating the inflowing gases (nitrogen and oxygen) from 0 to 60% air saturation at oscillation period of 300 s, in a complex medium containing whey, yeast extract, ammonium and magnesium sulphate.

The results reported in this paper proved that the set-up of a suitable medium represents a crucial aspect to be considered in the development of large-scale processes for β-galactosidase production, to further the range of its application and use.

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TABLE 4 - Time course of β-galactosidase activity of *Kluyveromyces* cells maintained on solid MW medium before growth in MW liquid medium containing raw cheese whey (Whey2), at 28 and 37 °C

Strain	Time (h)	EA_{spec} (IU/g dw)		EA_{max} (IU/ml)	
		28 °C	37 °C	28 °C	37 °C
CBS 2231	24	4851 ± 501	3151 ± 324	36.4 ± 4.0	44.1 ± 4.0
	48	2544 ± 225	3543 ± 302	44.0 ± 4.5	49.6 ± 5.0
	72	2263 ± 300	3368 ± 356	52.7 ± 5.0	58.9 ± 5.7
MIM 782	24	2919 ± 241	4353 ± 305	32.7 ± 3.5	37.4 ± 3.1
	48	2404 ± 197	3487 ± 354	40.6 ± 3.6	59.3 ± 6.0
	72	2313 ± 199	3184 ± 296	51.6 ± 5.9	66.5 ± 6.3
MIM 96	24	2393 ± 234	3996 ± 412	27.3 ± 2.3	48.0 ± 3.7
	48	2220 ± 200	2693 ± 287	37.7 ± 3.5	53.9 ± 5.1
	72	2144 ± 306	2956 ± 184	50.0 ± 4.8	60.1 ± 5.9

* EA_{spec}: specific activity, ** EA_{max}: volumetric activity.
Data are presented as mean ± standard deviation.

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