Convective Drying of the Thermotolerant *Kluyveromyces marxianus* at Relatively Low Temperatures and its Efficiency in Whey Fermentation

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Abstract: Thermally dried thermophilic *K. marxianus* has proved to be an effective starter culture for whey fermentation. Convective drying of *K. marxianus* can be performed effectively in the range 35-60^{o}C. The best drying temperature for is considered 35^{o}C since it is the most cost effective without any substantial difference in kinetic parameters when compared with higher temperatures.

The impact of thermally dried starter culture of *K. marxianus* is high, since several products could be produced from whey, such as potable and fuel-grade alcohol, baker's yeast, SCP to feed animals and a *Kefir* drink-type. Furthermore, the economical impact of thermally dried starter culture production is essential, since it may lead small dairy enterprises to treat their own whey by producing added value products and protecting the environment from this much polluted liquid. GC-MS analysis of fermented whey indicates that it contains volatiles similar to traditional drinks produced from vegetable raw materials.

INTRODUCTION

Whey is a major by-product of the dairy industry and its disposal without expensive sewage treatments represents a major source of water pollution due to the bulk quantities and its high organic matter content. Lactose is one of the main whey components responsible for its high Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) and can not be bio-conversed by the common yeasts of genus *Saccharomycces*. However, whey represents a potential substrate for a variety of microorganisms such as *Candida pseudotropicalis*, *Kluyveromyces marxianus* and *K. lactis*, etc., [1-4]. Therefore, the development of a highly productive process for fermenting lactose is of prime importance, as alcoholic fermentation can offer an alternative mode for bioremediation of whey [5].

K. marxianus is a yeast that produces β-galactosidase, enabling whey fermentation. This yeast offers great advantages, such as good growth yield, acceptability as a safe microorganism, higher β-galactosidase activity than other yeasts when lactose is used as substrate [6]. The importance of *K. marxianus* application in whey treatment in order to produce value added products, such as potable and grade-fuel alcohol, baker's yeast and SCP, raises the need for preservation and storage of large quantities of *K. marxianus* biomass. Several methods have been used for desiccating microorganisms, with freeze drying being the predominant among them. Although, freeze drying keeps the microorganisms viable for long periods and allows easy and inexpensive shipping, storing, and the high operational cost is preventive [7,8]. Another drying method applied for preservation of microorganisms is spray drying with a relatively lower cost, but with low viability and activity even for mesophilic lactic acid bacteria [9-12]. Furthermore, one of the special drying techniques is drying of bio-products with porous carriers. However, there are still no solid qualitative conclusions describing the effect of a porous carrier on quality retention of thermo labile materials [13]. Finally, convective air drying is a method that can be used for a wide range of biological products [14].

The aim of preserving cells by drying is to enable storage at ambient temperatures. Due to the inherent expense of chilled or frozen storage, reducing the storage temperature is not always an option to increase long term storage. Packaging methods such as storage under vacuum or in nitrogen may be a more cost effective way of regulating the storage environment [7].

The above presentation of literature manifests the need to investigate thermal drying of a thermo-tolerant microorganism at relatively low temperatures in order to develop a cost effective drying process. Therefore, the aim of the current study was to examine the application of thermal drying on the thermo-tolerant *K. marxianus.* Moreover, the effect of drying on viability and fermentation activity of *K. marxianus* was evaluated. Likewise, this drying process is compared to drying at ambient temperatures by using wheat flour as a porous carrier.

MATERIALS AND METHODOLOGY

Microorganism and Medium

K. marxianus IFO 288 culture was grown on whey at 30^{0}C under aerobic conditions by supplying air through a sterile filter. Wet *K. marxianus* cells were harvested at the late-exponential phase by centrifugation at 5,000 rpm for 10 min at 20^{0}C.

Synthetic medium consisting of 5% lactose, 0.1% ammonium sulphate, 0.1% mono-hydrogen potassium sulphate, 0.5% hydrated Magnesium sulphate and 0.4% yeast extract was prepared. It was sterilized in an autoclave for 15 min.

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1874-0707/07

Whey was produced in the laboratory from commercial milk, placed in a water bath at 37^{0}C, where 0.1 g/L of rennin was added. The mixture was left for 1 h and then the curd was cut in squares of 1 cm diameter and left to stand for 10 min. Whey was obtained by cloth filtration. The produced whey contained approximately 5% lactose and 0.8% proteins.

Drying of Biomass

Wet biomass of *K. marxianus* was distributed on a glass plate and was introduced in a convective dryer (J.P. Selecta, Spain), supplying successively hot air stream of 35, 42, 48, 55 and 60^{o}C. Drying kinetics was performed at each temperature. Drying was completed when constant weight was achieved.

Viability

1g of wet or reconstituted dried *K. marxianus* was subjected to serial dilutions with sterile ¼ strength ringer solution. Counts were taken by plating 0.1mL of each dilution on Potato Dextrose Agar (Fluka, Schweiz). Results are presented as the log of the mean number of CFU on solid-medium culture plates, containing between 30 and 300 colonies per g.

Fermentations

0.5g convectively dried *K. marxianus*, was added in a glass cylinder containing 300mL of sterile synthetic medium or whey, and the mixture was incubated at 33°C. The pH was adjusted at 5.5 by the addition of sulphuric acid and kept constant during fermentation, by the addition of sodium hydroxide solution 20%w/v at various time intervals. Fermentations were carried out, separately for convectively dried biomass at 35, 42, 48, 55 and 60^{o}C.

In addition, wet *K. marxianus* and corn flour in a ratio of 1:7, were stored at 4^{o}C for 4 months. The yeast formed granules when mixed with corn flour. After 4 months of storage, granules were sieved and were used for lactose and whey fermentations. Specifically, 0.5 g of yeast granules were used for fermentation of 300mL lactose synthetic medium and whey at 35, 45 and 55^{o}C.

Kinetics of fermentations was performed by lactose determination at various time intervals. At the end of fermentations, samples were collected and analyzed for ethanol, residual sugar, lactic acid and biomass concentration. Ethanol productivity is usually calculated in g/(1h). The ethanol yield factor is the ratio of g ethanol/g of utilised sugar during fermentation. Conversion is calculated using the equation: (Feed concentration-residual sugar) * 100/feed concentration.

Effect of Raisin Extract on Whey Fermentation by Convectively Dried *K. marxianus*

0.5g convectively dried *K. marxianus* and 3 mL black raisin extract of 12^{o}Be density were added in in a glass cylinder containing 300 mL whey and allowed to ferment The extract was prepared by extraction of 100 g black raisins with 200 mL tap water for at least 3h at 72^{o}C in an Erlenmeyer flask. Each mixture was incubated at 30°C until the end of fermentation. The pH value was adjusted during fermentation at 5.5 by the addition of sodium hydroxide solution 20%w/v. Kinetics was performed by analysing lactose at various time intervals. At the end of fermentations, samples were collected and analyzed for ethanol, residual sugar and lactic acid.

Assays

Lactic acid was determined by high performance liquid chromatography, using a Shimadzu chromatograph with a Shim-pack IC-A1 stainless steel column, a LC-10A pump, a CTO-10A oven at 40^{o}C and a CDD-6A conductivity detector. A solution of 2.5mM phthalic acid and 2.4mM Tris (hydroxymethyl) aminomethane (pH 4.0) in three times distilled water was used as mobile phase with a flow rate of 1.5mL/min. Samples of 0.25mL were diluted to 25mL and 60μL, and injected directly to the column. Lactic acid concentrations were calculated using standard curves.

Lactose was determined by high performance liquid chromatography, using a Shimadzu chromatograph with a SCR-101N stainless steel column, a LC-9A pump, a CTO-10A oven at 60^{o}C and a RID-6A refractive index detector. Three times distilled water was used as mobile phase with a flow rate of 0.8mL/min and 1-butanol was used as an internal standard. 0.5mL and 2.5mL of a 1% (v/v) solution of 1-butanol were diluted to 50mL, so as the actual concentration of 1-butanol was 0.05% (v/v). Then, 40μL of the final solution was injected directly to the column. Lactose and ethanol concentrations were calculated using standard curves.

Ethanol was analyzed by means of GC on a Shimadzu GC-8A instrument connected with a Shimadzu CR-6A integrator and an FID detector. The column was packed with Porapac-S and N_2 was used as carrier gas (40 mL/min). Column temperature was 140-180^{o}C (10^{o}C/min) and injection port and detector temperatures were 210^{o}C. 1-butanol (0.1 %v/v) was used as internal standard and samples of 4 μL were injected directly in the column. Determinations were carried out using both standard curves and internal standard method. The standard deviations for residual sugar and for ethanol were 0.2-0.5 and 0.3-0.5 respectively. Biomass concentration was determined by centrifugation of samples at 5,000 rpm for 10 min at 20^{0}C.

Volatiles by GC-MS Analysis

The headspace volatiles in samples of fermented whey were isolated by the solid-phase micro-extraction method (SPME). The fibre used was a 2cm-50/30mm DVB/Carboxen/PDMS StableFlex for manual holder (Supelco, USA). The conditions of headspace-SPME sampling used were as follows: 1 ml sample and 0.3g NaCl (saturated solution ~30%) were transferred into a 10 ml glass vial sealed with a rubber septum. The SPME fibre was exposed to the headspace and the vial was immersed in a waterbath at 60^{o}C for 1 h for absorption of volatiles. Desorption of volatiles took place in the injector of the gas chromatograph in splitless mode, at 250^{o}C for 3 min. GC/MS analysis was performed on a Shimadzu model GC-17A gas chromatograph coupled to a GCMS-QP5050A mass spectrometer. A Supelcowax-10 column with 0.25μm film thickness, 60m×0.32mm i.d. was used. The GC temperature program was 35^{o}C held for 5 min, then increased by 5^{o}C/min to 50^{o}C, where it was held again for 5 min, then increased by 5.5^{o}C/min to 230^{o}C, where it was held again for 5 min, for a total run time of 51.73min.

The carrier gas was helium with a column flow of 2mL/min. The injector was at 280°C in splitless mode. The interface temperature was 230°C. Mass spectra were recorded by electronic impact (EI) at 70eV. The scan mode was used to detect all the compounds in the range m/z 33-200. Identification of compounds was done by comparing the retention times and MS data with those of standard compounds and by MS data obtained from NIST107, NIST21 and SZTERP libraries. 4-methyl-2-pentanol was used as internal standard.

Scanning Electron Microscopy

The dried *K. marxianus* cells were monitored by scanning electron microscopy. The samples were coated with gold in a Balzers SCD 004 Sputter coater for 2 min and examined in a JSM-6300 scanning electron microscope at X3000.

RESULTS

Freeze drying has been used to preserve micro-organisms for decades and is the preferred method for culture collections worldwide [7]. However, due to its high cost, its substitution by a cost effective process such as thermal drying could be more profitable. Furthermore, because thermally dried microorganisms by spray drying have low viability, the scope was to examine a thermophilic microorganism useful for whey valorisation with the ability of lactose's bioconversion. In this case, the suitable thermophilic microorganism has to provide high impact in valorisation of whey due to whey's bulk global capacity. Therefore, the necessity of a microorganism leading to valorisation of whey and resulting in the production of various products, such as potable and fuel-grade alcohol, baker's yeast, SCP to feed animals and a *Kefir* drink- type product became apparent. Taking into consideration the above, the thermophilic *K. marxianus* was selected as the potential microorganism for convective drying. It has been stated that during Jerusalem Artichoke extract fermentation by free cells of *K. marxianus*, the optimum pH was around 5 and temperature was around 30-35°C, and the best biomass on lactose yield of *K. marxianus* cultivated on supplement whey permeate was for temperatures about 35°C and pH ranged at 4.5-5 [15,16].

Wet biomass was produced in an aerobic fermentor and drying was performed in a chamber with air supply at 35, 45, 55°C. Thermally dried *K. marxianus was* examined for lactose and whey fermentation efficiency. Our laboratory experience showed that the microorganism was able to resist infections.

In order to increase viability of cells and the fermentation efficiency, an alternative drying process at low temperatures was employed. The process comprises mixing of wet biomass with corn flour and storage for a long period, so that drying of cells by diffusion of water to hydroscopic starch, can be achieved.

Convective Drying of *K. Marxianus*

Fig. (**1**) shows that the increase of temperature does not reveal any rupture at the cell wall of the thermophilic yeast cells. Therefore, any death of yeast cells at various temperatures of convective drying is not originated from this, since the viability of thermally dried cells increased from 35 to 48°C and dropped at higher temperatures (Table **1**). Kinetics of drying at various temperatures, shown in Fig. (**2**), indicates drying at 55°C was completed within 1 hour, while at 35°C drying lasted at about 4 hours. This might explain the higher viability achieved at higher temperatures. The relatively low time necessary for a batch of convective drying at the optimum temperature of *K. marxianus*, is a positive result for the industrial application of the process.

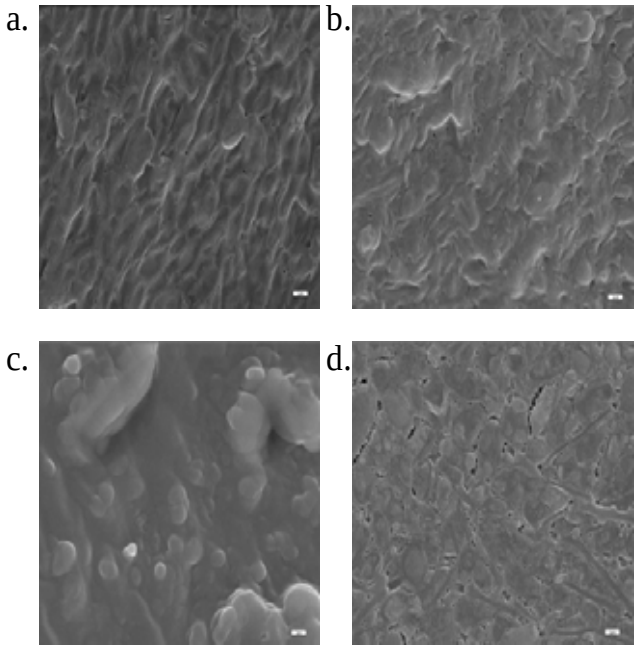


Fig. (1). Electron micrographs of dried *K. marxianus* cells with convective drying at (**a**) 35°C, (**b**) 42°C, (**c**) 48°C, (**d**) 55°C.

Validation of Fermentation Activity

In order to validate the fermentation efficiency of thermally dried *K. marxianus*, lactose and whey fermentations were performed at 35, 42, 48, 55 and 60°C (Fig. (**3**), Table **1**).

Both Figure and Table indicate that fermentations using whey led to much lower fermentation time and productivity than lactose. This can be attributed to the fact that whey is a more complete medium than the synthetic medium containing lactose. However, whey fermentations were completed in less than 100 hours and this result shows that drying in the range of 35-60°C is effective. Although drying at this range of temperatures resulted in duration of lactose fermentations from 100 h to 170 h, it did not happen in whey fermentation. In whey fermentation, the drying temperature did not play any significant role in fermentation efficiency eventhough viability was affected. In order to increase fermentation activity raisin extracts were used to promote whey fermentation. Fig. (**4**) shows that the addition of raisin extracts promoted whey fermentation, achieving 20% reduction in fermentation time. However, this achievement is much lower as compared to results obtained for whey fermentation using *Kefir* yeast [17].

Drying at Low Temperatures

Mixtures of wet *K. marxianus* and corn flour formed dried granules and those granules were selected and used for lactose and whey fermentation. The results are presented in Fig. (**5**). Whey resulted to faster fermentations as happened in case of thermally dried cells. Fermentation the times were

reduced as the temperature was decreased and in general are out of industrial applications, except of whey fermentations at 35°C.

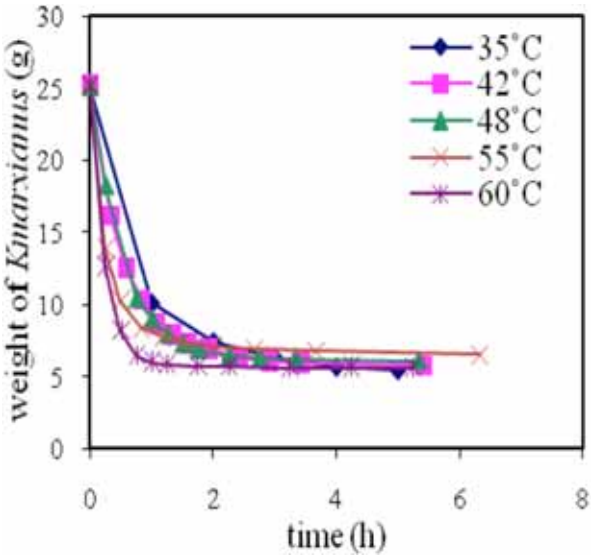
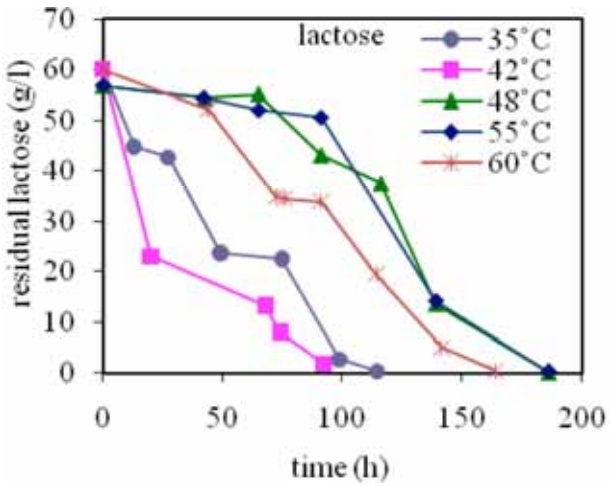


Fig. (2). Kinetics of convective drying of *K. marxianus* at various temperatures.

a.



b.

Formation of Volatiles

Fermented whey had to be examined since it is proposed to be for potable alcohol and stimulating *Kefir* drink production. The formation of volatiles were expected to be close to the chemical composition of fermented liquids, produced in industrial scale by traditional raw materials using strains of the genus *Saccharomycces*. Therefore, fermented whey products analysed by GC-MS and the results are presented in Table **2**. This analysis show, the increase of drying temperature increases the number of volatiles from 43 at 35°C to 48 at 42°C. This result is attributed to higher enzymatic activity of the thermophilic *K. marxianus* as the temperature is increased.

Drying at low temperature, lead to 48 total compounds. This result can be attributed to the presence of corn flour contains in addition to starting materials for bioconversion. More alcohols and esters are also produced at drying of 42°C and at low temperature drying in comparison with 35°C. The formation of more esters will have to contribute to improvement of the aroma of fermented products.

DISCUSSION

This investigation was organized in order to develop a cost effective starter culture production, for valorisation of whey and this is the reason for selecting convective drying of wet biomass of *K. marxianus* and its storage with corn flour. Fermentation times by convectively dried biomass were lower than the fermentations using *K. marxianus* stored with corn flour at low temperatures. These results make the decision for the selection of the best process to be convective drying of the thermophilic yeast. This decision also contributes to the increase of cost using corn flour for drying at low temperatures. The best temperature for drying is considered 35°C, due to this, above mentioned temperature is the most cost effective, without any substantial difference in kinetic parameters, in comparison with higher temperatures. Furthermore, the process included the addition of raisin extracts to improve the rate of fermentation, even-though the effect of raisin extracts was better in whey fermentation using *Kefir*. The impact of thermally dried starter culture of *K. marxianus* was high, since several products could be produced from whey. Except for the products that have been mentioned in the section of results, the starter culture of this thermophilic yeast held additional economical importance. The thermally dried starter culture production may contribute to small dairy enterprises to treat their own whey, producing added value products and protecting them from this much polluted liquid waste.

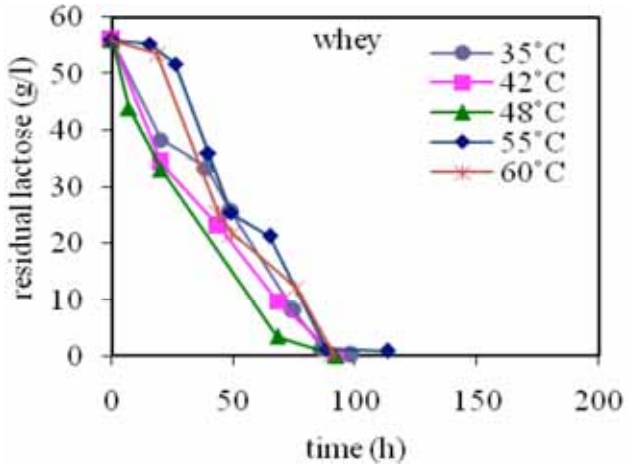


Fig. (3). Kinetics of fermentations at 33°C of synthetic medium containing lactose (**a**) and whey (**b**) using convectively dried *K. marxianus* at various temperatures.

It has to be stressed that whey fermented by thermally dried *K. marxianus*, contains various carbonyl compounds, higher alcohols, esters and organic acids, as are contained in fermented products using yeasts of the genus *Saccharomycces* and vegetable originated raw materials as grape

Table 1. Fermentation Kinetic Parameters of Lactose and Whey Using Thermally Dried Cells Incubated at Various Temperatures

	Temp (^{0}C)	Viability %	Alcohol Con. (%v/v)	Biomass (g/l)	Residual Lactose (g/l)	Fermentation Time (h)	Ethanol Productivity (g/l/h)	Ethanol Yield	Conversion %
Lactose	35	49.82	0.60	18.65	0.43	114	0.04	0.08	99.28
	42	73.78	0.60	18.18	1.95	92	0.05	0.08	96.75
	48	76.03	0.30	16.15	0.40	235	0.01	0.04	99.33
	55	60.92	0.17	15.35	0.01	235	0.01	0.02	99.98
	60	55.38	0.14	15.69	0.53	163.5	0.01	0.02	99.12
Whey	35	49.82	0.50	21.45	0.14	98.5	0.04	0.07	99.75
	42	73.78	0.42	21.36	0.01	92	0.04	0.06	99.98
	48	76.03	0.32	21.24	0.02	92	0.03	0.05	99.96
	55	60.92	0.26	22.33	0.90	113	0.04	0.04	98.39
	60	55.38	0.12	20.57	0.36	91	0.01	0.02	99.36

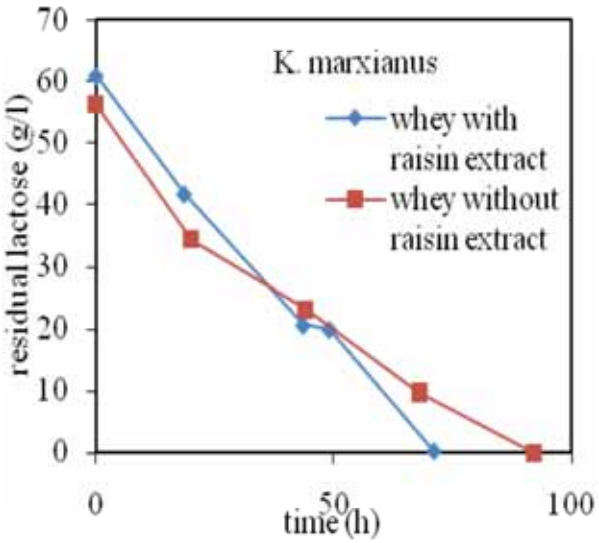


Fig. (4). Effect of raisin extract on kinetic of whey fermentation at 33^{o}C using convectively dried *K. marxianus* at 42^{o}C.

must, molasses and raisin extracts. However, some usual compounds such as acetaldehyde, propanol-1, iso-propyl alcohol, 2-methyl-butanol-1 and hexanol-1 are contained in wines and other drinks absent from these fermented products. This can be attributed to thermally dried *K. marxianus* due to fermented whey by freeze dried *Kefir* yeast contained in these compounds [18].

CONCLUSIONS

Convectively dried thermophilic *K. marxianus* is able to ferment whey with accepted fermentation time and productivity. Convective drying has to be preferred against drying at low temperatures, due to the cost of corn flour and kinetic data obtained. Drying at 35^{o}C, may have to be preferred, even-though less volatiles were obtained. Volatiles are contained in traditional drinks found in fermented whey. The absence of some of them such as acetaldehyde, propanol-1, iso-propyl alcohol, hexanol and 2-methyl butanol-1 will not worsen the quality of fermented products.

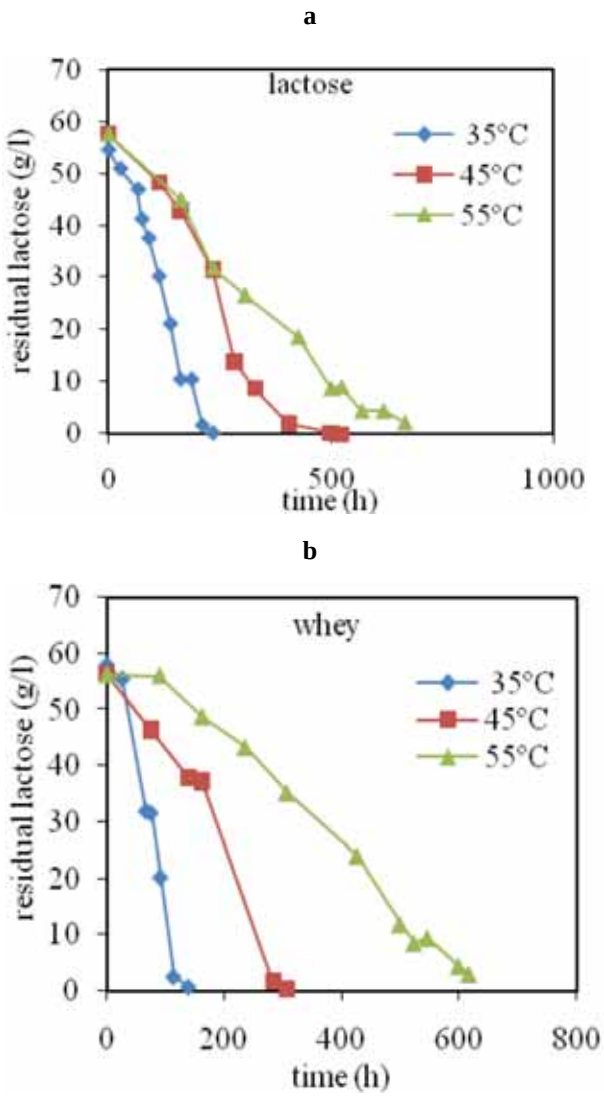


Fig. (5). Kinetics of synthetic medium containing lactose (**a**) and whey (**b**) fermentations at various temperatures using *K. marxianus* mixed with corn flour for 4 months storage.

Table 2. Volatiles Detected by GC-MS Analysis in Whey Fermentation at 33°C

Rt (Min)	Compound	Dried at 35°C	Dried at 42°C	Dried by Mixing with Corn Flour	Rt (Min)	Compound	Dried at 35°C	Dried at 42°C	Dried by Mixing with Corn Flour
Aldehydes(1)					39.492	1-dodecanol	n.d.	a	a
42.742	2-tetradecanal	a	n.d	a	41.767	1-tetradecanol	a	a	a
Total		**1**	**0**	**1**	42.750	2-undecanol	n.d	a	a
					43.317	9-tetradecen-1-ol	a	a	a
Ketones(11)					33.025	1-nonanol	a	a	a
15.675	2- Heptanone	a	a	a	43.692	1-hexadecanol	a	a	a
18.008	2-heptanone	n.d.	a	a	47.417	1-tetradecanol	n.d	a	a
25.275	2-nonanone	a	a	a	47.633	oxiraneethanol	a	n.d	n.d.
31.333	7-methyloctane-2,4-dion	n.d	n.d	a	**Total**		**19**	**23**	**20**
31.525	2-undecanone	a	a	a					
32.825	2-hexanone, 3-methyl-4-methylene	n.d	a	a	**Esters(13)**				
36.492	2,4-octanedione	a	n.d.	a	5.800	Ethyl acetate	a	n.d.	a
36.550	2-tridecanone	a	a	a	9.492	2-methyl-propylester acetic acid	n.d.	a	n.d.
40.925	2-nonadecanone	a	a	a	18.505	Hexanoic acid, ethyl ester	a	a	a
41.508	7-decen-2-one	a	a	a	26.783	Octanoic acid, ethyl ester	a	a	a
46.967	1-methoxy-3(-2-hydroxymet-hyl)nonane	a	a	a	30.017	Nonanoic acid, ethyl ester	n.d	a	a
Total		**8**	**9**	**11**	32.500	Decanoic acid, ethyl ester	a	a	a
					33.792	Ethyl 9-decenoate	a	a	a
Alcohols(25)					36.833	2-phenyl ethyl ester, acetic acid	a	a	a
6.967	Ethanol	a	a	a	37.225	Undecanoic acid, ethyl ester	a	a	a
13.925	2-methyl-1-propanol	n.d.	a	n.d.	41.408	Pentodecanoic acid, ethyl ester	a	a	a
14.917	2-methylacetate-1-butanol	n.d	a	n.d.	45.217	Octadecanoic acid ethyl ester	n.d.	a	n.d
14.992	3-methylacetate-1-butanol	n.d.	a	n.d.	45.225	Pentadecanoic acid, ethyl ester	n.d	n.d	a
17.492	4-methyl-2-pentanol	a	a	a	45.733	Ethyl-9-hexadecenoate	n.d	a	a
18.075	3-methyl-1-butanol	a	a	a	**Total**		**8**	**11**	**11**
22.975	2-heptanol	a	a	a					
29.408	2-nonanol	a	a	a	**Organic acids(8)**				
30.442	1-octanol	a	a	a	42.058	Octanoic acid	a	a	a
36.958	cyclodecanol	a	a	a	44.092	Nonanoic acid	a	a	a
37.292	2,6-octadien-1-ol, 3,7-dimethyl	a	a	a	44.167	n-hexadecanoic acid	a	n.d	n.d.
34.408	2-undecanol	a	a	a	45.883	n-decanoic acid	a	a	a
34.175	2-nonanol, 5-ethyl	a	n.d	n.d.	47.183	Nonanedioic acid	a	n.d	n.d.
35.400	1-decanol	a	a	a	50.117	Dodecanoic acid	a	n.d	a
38.775	2-tetradecanol	a	a	a	27.167	1-tridecene	a	a	n.d.
38.883	Phenylethyl alcohol	a	a	a	32.758	1-octadene	n.d.	a	a
39.483	cyclodecanol	a	a	a	**Total**		**7**	**5**	**5**
					Total compounds detected		**43**	**48**	**48**

a: compound detected
n.d: compound not detected

AKNOWLEDGEMENTS

This work has been performed within the framework of the Regional Operational Programme (ROP) of Western Greece and was co-funded by the European Regional Development Fund and the Region of Western Greece with finale beneficiary the Greek General Secretariat for Research and Technology.

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Received: June 29, 2007 Revised: August 6, 2007 Accepted: August 6, 2007