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Isolation and Molecular Identification of New *Kluyveromyces lactis* Strains Producing High Levels of Lactase and Invertase Enzymes

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Abstract

Six different new *Kluyveromyces lactis* strains were isolated from different dairy products from Bursa province, Turkey. Lactase and invertase enzyme activities of new *K. lactis* strains were determined under different growth conditions. Our results indicate that both lactase and invertase enzyme activities of new *K. lactis* strains are much higher than the standard *K. lactis* (ATCC8585). Lactase and invertase enzymes are expressed at high levels under glucose repressed growth conditions in some of these *K. lactis* strains. Invertase activities of new *K. lactis* strains are 10-fold higher than the standard *K. lactis* (ATCC8585) strain. Additionally, it was shown that the two of the new *K. lactis* strains have 25% shorter duplication times than the normal *K. lactis* (ATCC8585). Our results indicate that new *K. lactis* strains isolated from the local dairy products of the Bursa region of Turkey have distinct metabolic features with respect to the regulation of GAL/LAC regulon of *K. lactis*.

Keywords: *Kluyveromyces lactis*; Lactase; Invertase; Yeast identification; PCR-RFLP

INTRODUCTION

K. lactis is one of the industrial yeast strains that are used for lactose processing, enzyme and heterologous protein production [1, 2]. Lactase enzyme (β-Galactosidase, E.C. 3.2.1.23) produced mainly by *K. lactis* is also important industrial enzyme that is used for the production of galacto-oligosaccharides and lactulose as a reaction products under specific reaction conditions. Both lactulose and galacto-oligosaccharides are highly recommended food additives that improve the growth of prebiotic bacteria [3, 4].

Lactase enzyme encoded by *KlLAC4* gene of *K. lactis* and its expression is regulated by glucose repression mechanisms [5, 6]. Hence expression of lactase depends on the growth conditions (e.i. lactose, glucose concentrations in the growth medium) [7, 8]. Moreover, expression level of lactase in *K. lactis* is a highly strain dependent phenomenon [9]. Transcription of *KlLAC4* is regulated by DNA binding transcription factor KlGal4 [10]. An activity of KlGal4 is controlled by the regulatory factors KlGal1 and KlGal80. When KlGal1 and KlGal80 interacts in the presence of lactose or galactose, KlGal4 relives from repression and activates the transcription of *KlLAC4*, resulting in high level expression of lactase (Anders et al. 2006). Therefore, analysis of the structure and expression patterns of the GAL/LAC genes in new *K. lactis* strains has significant implications in yeast biotechnology [11, 12].

Invertase is another enzyme that is used in sucrose processing in various industrial fields [13]. Invertase enzyme encoded by *KlINV1* gene in *K. lactis* and its expression also regulated by glucose repression [14]. However, regulatory factors involved in the control of invertase expression from *KlINV1* have not been reported in details yet.

Natural dairy products are the major resources for the isolation of lactose metabolizing industrial microorganisms. Yeasts flora in particular dairy products is one of the main components of the ripening process and development of characteristic tastes. In addition, certain species of yeasts may also cause spoilage of dairy products resulting in significant economic loss [15, 16]. Yeast species belonging to *Debaryomyces*, *Geotrichum*, *Candida*, *Pichia*, *Kluyveromyces, Yarrowia* and *Rhodotorula* genera can be found in different dairy products [15, 17, 18]. *Kluyveromyces lactis* is the well-known "milk yeast" that is present in almost all of milk samples in varying frequencies [1, 19].

The aims of this study are to screen local milk and cheese samples to isolate new *K. lactis* strains that might be used for the industrial purposes. Hence, Milk and cheese samples were collected from the local dairies and analyzed for the new *K. lactis* strains. Invertase and lactase activities of newly isolated *K. lactis* MY strains were analyzed in different growth conditions.

MATERIALS AND METHODS

Isolation and identification of yeast strains

The yeast strains were isolated from different cows raw milk and cheese samples obtained from 5 different local dairy producers in Bursa province, Turkey. 20 ml of raw milk samples were taken into sterile falcon tubes immediately after production. Feta cheeses samples (10-50 g), freshly made only with animal rennet from raw milk and ripened 5 days, were also taken into sterile containers to be processed in the laboratory.

To analyze and determine the yeast flora, 50 μl of aliquots from milk samples directly spread on YGC (Yeast Extract Glucose Chloramphenicol Agar) plates [15]. Cheese samples (0.5 g) were first totally homogenized in 10 ml of 2% sodium citrate solution and then 50 μl of this cheese homogenate samples were spread on YGC plates [15]. Plates were incubated at 30 °C for 3 days and yeast colonies counted for determination of colony forming units (CFU) of milk and cheese samples. To identify the species of the yeasts grown from the milk and cheese samples, 150 well isolated yeast colonies randomly selected from the primary YGC plates and patched on fresh YPD (1% yeast extract, 2% bacto-peptone, 2% glucose, 2% agar agar) plates. Yeast patches were incubated at 30 °C incubator for 3 days. From these 150 yeast samples, 40 well-grown yeasts were screened for species identification and lactose positive phenotype. Species identification for yeast samples were done first with API32C kit (Biomerieux) as described by the manufacturer [20].

Genetic characterization of *K. lactis* strains

In order to investigate the regulation of lactase and invertase activities, 6 different *K. lactis* strain (named as *K. lactis* MY22-25, MY28, and MY29) were selected from the isolated lactose positive yeast strains. Genomic DNA was isolated from *K. lactis* strains [21]. In addition to API32C tests, species of new *K. lactis* strains were also identified by sequencing of ITS1-5.8S rDNA-ITS2 region of ribosomal DNA as described by White et al. [22] using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers and same PCR conditions. PCR products were purified by PCR product purification kit (Roche) and sequenced directly by DYEnamic ET terminator cycle sequencing kit (Amersham) and ABI PRISM 310 Genetic Analyzer. BLAST analysis (NCBI) of the nucleotide sequences of ITS-5.8S rDNA regions of new *K. lactis* strains identified these yeasts as 100 % *K. lactis*. To further confirm the species identities, Ribosomal DNA Restriction Fragment Length Polymorphism (PCR-RFLP) analysis of ITS-5.8S rDNA regions of isolated *K. lactis* strain were also done with Hinf1 enzyme as described [23]. Previously defined *K. lactis* strain (ATCC8585) was used as a standard control strain in all of our experiments.

Analysis of lactase and invertase activities

Lactase activities of isolated *K. lactis* strains were determined in permeabilized yeast cells as described [7]. Briefly, *K. lactis* strains were grown overnight at 30 °C incubator shaker (140 rev/min) in 5 ml of YP (1% yeast extract, 2% bacto-peptone) medium supplemented with different carbon sources (4% glucose or 2% lactose) to obtain saturated pre-cultures. Then these cultures were used to inoculate fresh 5 ml YP mediums supplemented with 4% glucose or 2% lactose as carbon sources. The initial cell densities of these fresh cultures were adjusted to OD_{600}: 0.2. *K. lactis* cultures were grown to mid-logarithmic stage (OD_{600}: 1.0) at same growth conditions. At the end of growth periods, *K. lactis* cells were harvested by centrifugation and washed once with 1 ml of ice-cold sterile distilled water and resuspended in 200 μl of breaking buffer [21]. Yeast strains were grown in duplicates in all enzyme assays. Lactase assays were done in triplicates and repeated at least once under same experimental conditions. Mean lactase activities of *K. lactis* strains were expressed as Miller Units [21].

In order to determine the secreted invertase activities, *K. lactis* strains were pre-cultured in 5 ml of YPD medium overnight at 30 °C incubator shaker (140 rev/min). These saturated pre-cultures were used to inoculate fresh 10 ml of YPD medium in duplicates. Initial cell densities of the yeast cultures were adjusted to OD_{600}:0.2 and grown to logarithmic stage under standard conditions. Then, a portion of yeast cultures (5 ml) was harvested, washed with 5 ml of sterile distilled water twice and shifted to 5 ml of derepressed growth medium (YP+0.05% glucose) for 2 hours [24]. The secreted invertase activities of the *K. lactis* strains were determined using whole cells in duplicates as described [25, 26]. Invertase activities were expressed in μmoles of glucose deliberated per minute per 100 mg (dry weight) of *K. lactis* strains at 37 °C [24].

Determination of growth rates

Duplication times of *K. lactis* strains were determined in yeast cultures grown in 20 ml YPD medium in standard growth conditions (30 °C incubator shaker at 140 rev/min speed). Initial cell densities of yeast cultures were adjusted to OD_{600}:0.2 by inoculations from saturated cultures as described in enzyme assays. *K. lactis* cultures were incubated in standard growth conditions for 16 hours. OD_{600} values of each yeast cultures were measured every 90 minutes. Yeast cultures were grown in duplicates and duplication times were determined graphically by plotting mean OD_{600} values of yeast cultures versus time points.

RESULTS

Analysis of yeast diversity in the dairy products

Milk and cheese samples obtained from local dairies of the Bursa region of Turkey showed highly diverse yeast flora. Yeast counts in the milk samples vary between $2x10^3$ to $8x10^3$ CFU/ml. Yeast counts was determined as $\approx 2x10^4$ CFU/g in the cheese samples, being at least 10-fold higher than the yeasts in milk samples. In total, 40 different well-grown yeast specimens were screened for species identification with API32C yeast species identification system. In these yeasts, 10 different yeast species were identified. The most frequent yeast species found in local milk and cheese samples was *Kluyveromyces marxianus* (24%). Other abundant yeast species are *Pichia fermentas*, *Candida colliculosa* and *Kluyveromyces lactis*. The frequencies of these species are approximately 16-19%. Less abundant yeast species identified in milk and cheese samples are *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*, *Candida zeylanoides*, *Candida crusei*, *Candida lusitaniae* and *Cryptococcus albidus*. Frequencies of these yeast also varies between 3-5% in milk and cheese samples.

In order to further confirm and validate the species of newly isolated *K. lactis* strains, genomic DNA was isolated from these yeasts and ITS-5.8S rDNA region were amplified with PCR as described [22]. ITS-5.8S rDNA regions of all *K. lactis* strain yielded approximately 720 bp long PCR amplicon, which is characteristic for *K. lactis*

[23, 27]. Amplified regions were sequenced and then subjected to BLAST analysis. Results of BLAST analysis revealed that the isolated *K. lactis* strain are indeed *K. lactis*. Restriction of ITS-5.8S-rDNA regions of isolated *K. lactis* strains by Hinf1 generated 4 DNA fragments [23, 27] (Fig. 1). The sizes of Hinf1 generated DNA fragments are the same as in control *K. lactis* strain. The lengths of these DNA fragments were reported as 297±4 bp, 195±5 bp, 128±3 bp and 91±1 bp (Fig. 1) [23, 27]. This result further confirmed that the isolated strains are *K. lactis* and they are named as *K. lactis* MY (Milk Yeast) strains.

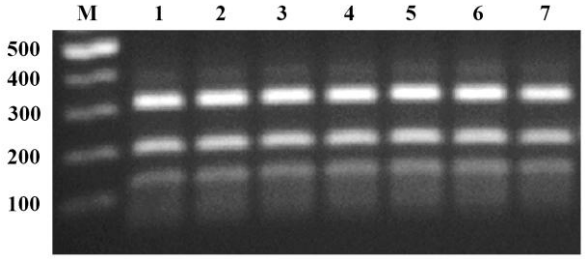

Figure 1. PCR-rRFLP analysis of *K. lactis* strains ITS-5.8S rDNA regions. Genomic DNA were isolated from *K. lactis* strains, ITS-5.8S rDNA region PCR amplified and subjected to Hinf1 digestion. M: Marker DNA, 1: Standard *K. lactis* (ATCC8585), 2-7: *K. lactis* strains MY22-25 and MY28-29, respectively.

Analysis of lactase activities of the isolated *K. lactis* strains

Transcription of lactase enzyme from its structural gene *KlLAC4* is regulated by glucose repression and derepression mechanisms [5, 6]. Hence, *K. lactis* samples were grown both in repressed and derepressed growth conditions. Growth of *K. lactis* strains in lactose medium yielded 550 to 721 Miller Units (MU) of lactase activity in newly isolated *K. lactis* strains (Table 1). The highest lactase activity (721±22 MU) measured in *K. lactis* MY24, while the lowest lactase activity (553±4 MU) is in *K. lactis* MY25 strain. Lactase activity of control *K. lactis* strain was measured as 735 MU in lactose medium (Table 1).

Table 1. Lactase activities of *K. lactis* MY strains.

	Lactase Activities[a]	
***K. lactis* Strains**	**YP + 2% lactose**	**YP + %4 Glucose**
K. lactis (MY 22)	617 ±6	124 ±2
K. lactis (MY 23)	653 ±55	112 ±2
K. lactis (MY 24)	721 ±22	117 ±1
K. lactis (MY 25)	553 ±4	112 ±12
K. lactis (MY 28)	573 ±48	93 ±6
K. lactis (MY 29)	633 ±28	127 ±8
K. lactis (ATCC8585)	735 ±12	67 ±21

[a]Lactase activities were given in Miller Units (± Standard deviations).

To test the effects of glucose repression on the expression of lactase, *K. lactis* strains were grown in high glucose medium (4% glucose) and lactase activities were determined at logarithmic stage. Lactase expression in the control *K. lactis* strain decreased about 11-fold (from 735 to 67 MU). However, the effects of glucose repression on lactase expression in *K. lactis* MY strains were a lot lower than the control strain. Decrease in the lactase activities of *K. lactis* MY strains grown in glucose was approximately 5-fold. Lactase expression was approximately 2-fold higher (124 to 127 MU) than the control strain in *K. lactis* MY22 and MY29 strains of *K. lactis* under glucose repressed growth conditions.

Analysis of invertase activities of *K. lactis* strains

Analysis of secreted invertase enzyme activities of *K. lactis* MY strains indicated that expression of invertase is less sensitive to glucose repression. Under glucose repressed growth conditions, invertase activity of control *K. lactis* (ATCC8585) strain measured as 49 units (Table 2). However, invertase activities of the newly isolated *K. lactis* MY strain were 5 to 7-fold higher than the standard *K. lactis* (Table 2). Invertase activities of *K. lactis* MY strains vary from 230 units (*K. lactis* MY23) to 353 units (*K. lactis* MY25) in glucose repressed growth condition (Table 2).

Shifting of *K. lactis* strains to glucose derepressed growth conditions resulted in the activation of invertase expression as expected. Derepressed level invertase activity of standard *K. lactis* strain identified as 154 units. Invertase activities of *K. lactis* MY strains also derepressed and increased up to 622 units. Derepressed level invertase activities of *K. lactis* MY strains were 3-4 fold higher than the standard *K. lactis* (ATCC8585) strain. The highest invertase activity measured in *K. lactis* MY28 strain, while the lowest activity was in *K. lactis* MY29 strain. The invertase activities of other *K. lactis* MY strains varied from 523 units to 573 units (Table 2).

Table 2. Invertase activities of *K. lactis* MY strains.

	Invertase Activities[a]	
***K. lactis* Strains**	**Repressed**	**Derepressed**
K. lactis (MY22)	325 ±11	537 ±35
K. lactis (MY23)	230 ±29	523 ±4
K. lactis (MY24)	319 ±81	573 ±3
K. lactis (MY25)	353 ±39	561 ±14
K. lactis (MY28)	309 ±71	622 ±95
K. lactis (MY29)	321 ±21	472 ±27
K. lactis (ATCC8585)	49 ±6	154 ±1

[a]invertase activities were given in μmoles of glucose deliberated per min per 100 mg (dry weight) of cells (± Standard deviations).

Determination and evaluation of the duplication times

Growth rate is one of the most important industrial features of the yeasts. Yeasts with shorter duplication times are preferred for biomass production. Hence duplication times at logarithmic stage of the *K. lactis* MY strains were determined by growing yeast cultures in standard conditions (30 °C, 140 rev/min). Duplication time of

Table 3. Duplication times of *K. lactis* MY strains.

***K. lactis* strains**	**Duplication Times[a]**
K. lactis (MY22)	110 ±9
K. lactis (MY23)	112 ±3
K. lactis (MY24)	126 ±6
K. lactis (MY25)	90 ±1
K. lactis (MY28)	76 ±3
K. lactis (MY29)	138 ±33
K. lactis (ATCC8585)	120 ±6

[a]Duplication times were given in minutes for logarithmically growing yeast cells (± Standard deviations).

standard *K. lactis* (ATCC8585) strain was measured as 120 min. Duplication times of *K. lactis* MY22- 24, and MY29 were approximately same as the control *K. lactis* strain. A faster growth and hence shorter duplication times were measured for the *K. lactis* MY25 and MY28 strains. Duplication time of *K. lactis* MY25 strain was 25% shorter (90 min) than the standard strain. Duplication time of *K. lactis* MY28 strain was identified as 76 min (37% shorter than the standard *K. lactis*).

DISCUSSION

K. lactis is an important industrial yeast species. It is widely used in the production of lactase enzyme and lactase is used for processing of lactose containing food stuff [1, 11]. In addition, *K. lactis* has a great potential for the production of therapeutic peptides due to its superior secretion system and growth characteristics [2, 28]. To isolate new *K. lactis* strains with high lactase and invertase activities we have screened milk and cheese samples from the local dairies of Bursa. Among the lactose positive yeast samples, 6 *K. lactis* strain selected for further metabolic characterization.

Both the number and the species distribution vary greatly in the dairy products. This diversity mostly depends upon the source of milk and processing methods. Total yeast counts in the milk and cheese samples analyzed in this study ranges from $2x10^3$ to $2x10^4$ CFU/ml milk or g/cheese, respectively. These values are close to the previously reported yeast counts in the milk products [18, 29]. Total yeast counts in the dairy samples can go up to 10^7 CFU/ml milk [29]. Cosentino et al. [17] screened 150 cheese samples for yeast diversity and identified 25 different yeast species. We have identified 10 different species in the 40 yeast samples tested for species idenfication in milk and cheese samples used in this research. Species diversity seems to be reasonable in milk and cheese samples analyzed in this research. The most frequent species identified as *K. marxianus* (24%). The frequency of *K. lactis* strains is 16%. Dominating yeast species also shows great differences depending on dairy products. In certain cheese samples, *K. lactis* is the major yeast while it is *D. hansenii* in other cheese samples [17, 18]

Lactase is one of the most significant industrial enzymes that is synthesized by *K. lactis*. Hence, *K. lactis* strain that continuously produces high level of lactase has important applications in the processing of lactose containing products [11, 12]. The lactase activities of *K. lactis* MY strain showed distinct features when compared to standard *K. lactis* (ATCC8585). It is known that the expression of lactase enzyme is regulated by glucose repression. Lactase activities of *K. lactis* MY strain is close to the standard strain's level when grown in lactose medium. However, lactase activity of *K. lactis* MY strain is 2-3 fold higher than the standard strain under glucose repressed growth conditions. This result shows that the lactase expression is more resistant to glucose repression than the standard strain. Regulatory factors that involve in the expression of lactase are KlGal4, KlGal1 and KlGal80 [30]. Previously, Kuger et al [31] also isolated *K. lactis* strains that are resistant to glucose repression and identified mutations in the zinc finger domain of *KlLAC9* (KlGal4) gene. Suleau et al. [32] compared the expression patterns of GAL/LAC genes of two *K. lactis* strains by microarray analysis by growing them in glucose or lactose medium. They have identified significant differences in the expression patterns of these genes by microarray analysis [32]. We have cloned and sequenced the KlGAL4 gene of K. lactis MY strains. We could not identified any mutations neither in DNA binding nor in activation domain of KlGAL4 of *K. lactis* MY strains. Further investigations needs to be done on the expression patterns of GAL/LAC genes of *K. lactis* MY strains to uncover the molecular reasons for the high levels of lactase expression in glucose medium.

Invertase activities of *K. lactis* MY strains are much higher than the standard strain both in repressed and derepressed growth conditions. Invertase enzyme of *K. lactis* is encoded by *KlINV1* gene [14]. Transcription factors involve in the expression of invertase in *K. lactis* has not been elucidated in details yet. Unlike *SUC2* gene of *S. cerevisiae*, it is known that the transcription of *KlINV1* is independent of repressor protein Mig1 [14]. *K. lactis* MY strains might be a good models for the identification of the regulators of *KlINV1* gene expression in *K. lactis*.

A duplication time of the yeast strains depends on their ability to use carbon courses in the growth medium. It is known that the faster consumption of lactose results in the shorter duplication times and it depends on the expression of GAL/LAC regulon in *K. lactis* [33]. Based on this fact, effective utilization of carbon sources may result in faster growth of *K. lactis* MY25 and MY26 strain which has shorter duplication times than the others.

In conclusion, we have isolated and defined new *K. lactis* strains from local dairy products that show distinct expression patterns for lactase and invertase enzymes. The duplication times of the two of newly isolated *K. lactis* strain are much shorter than the standard *K. lactis*. These new *K. lactis* strains can be developed for industrial uses such as lactase and invertase production in whey or in different molasses.

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