Molecular Genetic studies on the natural yeasts isolated from Egyptian dairy products.

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Abstract:

Cheese whey is a by-product of dairy industries, produced in large amounts and has a high polluting load, therefore representing a significant environmental problem all over the world. The lactose in cheese whey can be used as a substrate for bioethanol production by microbial fermentation. Its anaerobic digestion offers an excellent approach in terms of both energy conservation, pollution control, and represents the least expensive carbon source for ethanol production. The present study aimed to detect the potent yeast strains that ferment the lactose in cheese whey which were isolated from whey samples collected from different geographic regions in Egypt. Thirty nine yeast isolates were isolated from which four strains (Z-2, Z-5, Z-13 and Z-39) were selected based on their fermentative activity, and lactose tolerance. Results of lactose tolerance indicated that the selected strains Z-2, Z-5 and Z-13 showed normal growth at concentrations of 2% and 10%, then showed lower ability to grow at concentrations up to 60%. On the other hand strain Z-39 exhibted moderate growth only at 2% while it couldn't be observed at other concentrations. The selected isolates were identified at the molecular level as *Pichia sp.* (Z-2), *Kluyveromyces sp.* (Z-5), *Kluyveromyces sp.* (Z-13) and non-Saccharomyces strain (Z-39). Thereafter, RAPD-PCR for the four strains was performed using five different primers, the results of DNA fingerprints exhibited distinct band profiles indicating a clear differentiation of the four strains. Selected strains were regrouped in two clusters; one of them included Z-5 and Z-13, and the second cluster contained Z-2 and Z-39.

Introduction:

Cheese whey is a by-product of dairy industries which is produced as a waste stream from dairy production facilities. This by-product of cheese production represents about 85-95% of the milk volume and retains 55% of milk nutrients, such as lactose, soluble proteins and lipids (Becerra, *et al.*, 2006).

The high volumes of cheese whey that are produced from dairy industries, in addition to its

high organic matter control, it can lead to a considerable environmental problem. On the other hand, the cheese whey has a high biological oxygen demand (BOD) 40.000- 60.000 ppm when dumped in sewers or disposed of on the land (Ghaly and Kamal, 2004).

The relatively high content of lactose in cheese whey does indeed suggest the possibility of bio-conversion of lactose into ethanol by yeast (Sansonetti, *et al.*, 2010). *Saccharomyces cerevisiae*, the yeast most utilized in fermentations, lacks the lactose permease system as well as the intracellular enzyme for lactose hydrolysis, β-galactosidase, thus rendering it unable to ferment lactose directly into ethanol (Russel, 1986; Castillo, 1990; Siso, 1996).

Kargi and Ozmıhcı, (2006) reported that most of the *Kluyveromyces* species are capable of fermenting lactose present in cheese whey to ethanol. However, Zafar and Owais, (2006) showed that it is very important to choose a strain with suitable physiological characteristics to achieve a good utilization of lactose from whey.

Consequently, dairy products represent a specific environment for the growth and selection of different yeast species (Fleet, 1990; Viljoen, 2001; Lopandic, *et al.*, 2006).

The progress in molecular biology during the last decades opened the possibility to charac-terize yeasts at the genomic level. One such method is a variant of the polymerase chain reaction (PCR) technique based on random amplified polymorphic DNA (RAPD). RAPD technique relies on the use of arbitrary primers which are annealed to genomic DNA using low temperature conditions (Williams, et al., 1990; Welsh and McCleland, 1991; Angela, *et al.*, 2000; Lather, *et al.*, 2010). Priming at a number of closely adjacent complementary sites allows the subsequent amplification of dispersed genomic sequences by taq DNA polymerase enzyme (Valério, *et al.*, 2006). This technique detects genetic polymorphisms and does not depend on prior knowledge of species-specific sequences.

Couto, *et al.*, (1994 and 1995) indicated that in RAPD technique the level of differentiation, either inter-or intraspecies depend highly on the primers used. The RAPD assay, using selected 10-mer oligonucleotides, allowed the discrimination between all species tested (Couto, *et al.*, 1994).

The current investigation aimed to isolate lactose fermenting yeast strains from Egyptian dairy products to produce bio-ethanol and the recent molecular genetic techniques (RAPD) were carried out to differentiate the selected yeast strains at the molecular level.

Materials and Methods :

  Sampling

Samples of cheese and yogurt whey were collected from dairy producing centers in different geographical regions in Egypt and used were for the isolation of naturally occurring yeast strains. 100 µl of each whey sample, were spread over the surface of plates containing YEPD medium (0.5% yeast extract, 2% peptone, 2% dextrose, pH 4.5) supplemented with 0.1 gl^{-1} chloramphenicol. The plates were incubated at 28 – 30°C for 48 h. After incubation, colonies with different morphological characters were selected, examined using light microscope and purified by streaking on the same medium. The purified isolates were stored on YEPD slants at 4°C (Nahvi and Moeini, 2004).

Yeast Evaluation and Selection

Litmus milk and acid formation method was used as an indicator for ethanol production due to lactose fermentation by yeast. Tubes containing Litmus milk media were inoculated by a loopfull of Yeast isolates. Tubes were incubated at 28°C and observed for colour changing from blue to pink each 1h and the fast isolates were selected.

Lactose tolerance

Lactose tolerance was examined on agar plates containing yeast extract peptone supplemented with lactose (YEPL) at different concentrations: 2, 10, 40 and 60%. A 100 µl from 10^{-4} dilution of selected isolates were spread on the agar plates. The plates were incubated at 28 –

30°C for 96 h followed by counting the number of viable colonies, (Senses-Ergul, *et al.*, 2007).

Isolation of genomic DNA for PCR:

Cells used for DNA extraction were grown for approximately 24 h at 25°C in 50 ml YEPD broth medium on a rotary shaker at 200 rpm and harvested by centrifugation. The cells were washed once with distilled water, re-suspended in 2 ml of distilled water, and the suspension was divided into two 1.5 ml microcentrifuge tubes. After centrifugation, the supernatant was decanted, and the pillets were used for DNA extraction which was performed according to Harju *et al.*, (2004) and Hesham, *et al.*, (2009) procedures

PCR amplification and gel run

RAPD profiles were generated using five decamer primers (OPA-2, OPA-3, OPA-9, OPA-11, OPA-15) (Martorell, *et al.*, 2005) as shown in table (1). The PCR was performed in a final volume of 25 µl containing 11.0 µl dH$_2$O, 3.0 µl 10X reaction buffer, 3.0 µl dNTP's mix, 2.0 µl Primer, 1.0 µl Taq polymerase, 4.0 µl MgCl2 and 1.0 µl Template DNA.

The amplification reactions were carried out in a thermocycler under the following conditions: initial denaturation at 94°C for 5 min, followed by 45 cycles of 92°C for 1 min, 36°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 10 min and subsequently cooled to 4°C. Ten

μl of the amplified mixture was then analyzed using 1.5% 0.5×TBE agarose gel electrophoresis (Martorell, *et al.,* 2005).

The gel was stained with ethidium bromide 0.01%, visualized under UV light, and photographed. The molecular sizes of DNA fragments were obtained with comparison with a 100-bp molecular marker.

Table (1) PCR primers used in the present study

Target DNA	Primers Operon/Design	PCR primers sequence 10-mer in length - 5' to 3'	Annealing temperature (°C)
Genomic DNA	OPA-02	TGCCGCGCTG	36
Genomic DNA	OPA-03	AGTCAGCCAC	36
Genomic DNA	OPA-09	GGGTAACGCC	36
Genomic DNA	OPA-11	CAATCGCCGT	36
Genomic DNA	OPA-15	TTCCGAACCC	36

RAPD analysis

Agarose gel photos were scanned by the Gene Profiler 4.03 . A binary data matrix recording the presence (1) or the absence (0) of bands was made. The software package MVSP (Multi-Variate Statistical Package) was used and genetic similarities were computed using the Dice coefficient of similarity (Nei and Li, 1979):

$$Similarity = \frac{2 * n11}{(2 * n11) + n01 + n10}$$

Where:

n11 - designates the number of common bands for the two compared samples,

n10 - cases where the bands were visible only in the first sample,

n01- when bands were visible in the other sample only (Dice, 1945).

Cluster analysis was carried out on similarity estimates using the unweighted pair-group method with arithmetic average (UPGMA) software. These methods were carried out using MVSP software programs as in (Youssef, 2004). The results were then represented as a dendrogram for allover primers. Data analyses and dendrogram diagrams were also carried out.

Results and Discussion:

Isolation and screening of lactose fermenting yeasts

Yeasts in the Egyptian dairy products showed great phenotypic biodiversity. A total of 39 yeast isolates were obtained from various dairy products collected from different geographical re-

gions in Egypt. All isolated yeast strains were tested for colour changes of litmus milk. Four isolates were selected, three of them were able to change color fast to pink after 3h of incubation as an indicator for acid formation, which was referring to the potence of the strains for lactose fermentation, whereas the fourth one couldn't do any change (Figure 1).
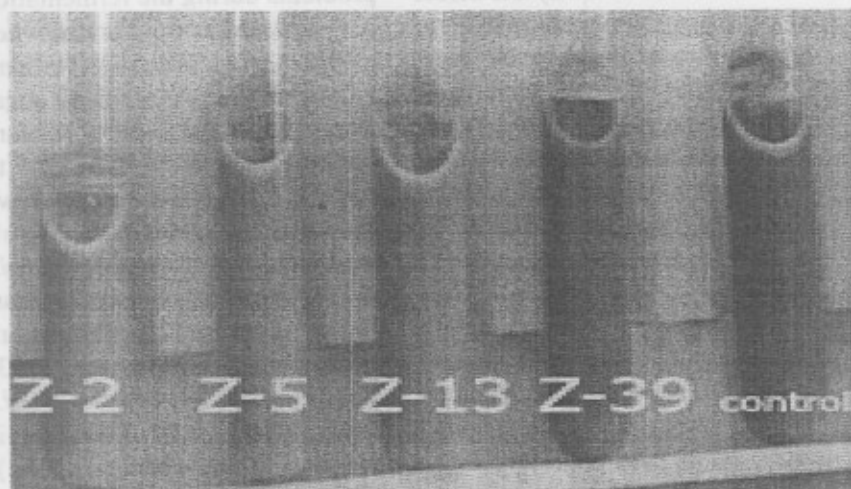

Figure (1). Hot Pink color shows acid production - indicating lactose fermentation

Table (2) shows the characterization of the selected yeast strains, including their origin and their molecular identification.

Table (2). Characterization of the selected yeast strains

No.	Source	Location	Code	Genus
1	local Cheese	Gerga	Z-2	*Pichia sp.*
2	local Cheese	Qena	Z-5	*Kluyveromyces sp.*
3	local Yoghurt	Nag. Hammadi	Z-13	*Kluyveromyces sp.*
4	Manufacture Yoghurt (Goy)	Goy, Cairo	Z-39	Non-Saccharomyces

Several studies have shown the wide biodiversity in yeast population of dairy products worldwide, (Pereira-Dias, *et al.*, 2000; Álvarez-Martín, *et al.*, 2007, Capece and Romano, 2009). El-Sharoud, *et al.*, (2009), studied and examined the diversity and ecology of yeasts associated with traditional Egyptian dairy products.

Although the yeasts that assimilate lactose aerobically are widespread, those that ferment

lactose are rather rare (Fukuhara, 2006), including e.g. *Kluyveromyces lactis*, *K. marxianus*, and *Candida pseudotropicalis*. (Guimarães, *et al.*, 2010).

Lactose Tolerance

In order to study the effect of lactose concentrations on growth rate of the selected yeast isolates, the YEP medium supplemented with different concentrations of lactose 2, 10, 40 and 60% (v/v) were used and counted according to (Senses-Ergul, *et al.*, 2007). As shown in Table (3) Strains Z-2, Z-5 and Z-13 revealed good and moderate growth on 2% and 10% respectively, followed by a lower ability to grow at concentrations above 40%. On the other hand strain Z-39 showed moderate growth only on 2% while did not show any growth at other concentrations.

In order to reduce distillation costs during bio-ethanol production, it is desirable to obtain high ethanol concentration during fermentation, starting with high sugar concentrations. This objective can be obtained if the yeast has the ability to tolerate high lactose concentration. Previously, very few types of yeasts were known to tolerate sugar concen-

trations of above 40% and normally at such concentrations, yeasts grew very slowly (Benitez, *et al.*, 1983; Sumari, *et al.*, 2010). Many authors have reported inhibitory effects and associated problems during the fermentation of concentrated lactose/whey media, particularly slow fermentations and high residual sugar when the initial lactose concentration is increased above 100 to 150 g L^{-1}, or in some cases above 200 g L^{-1} (Gawel and Kosikowski, 1978; Janssens, *et al.*, 1983; Vienne and von Stockar, 1985; Kamini and Gunasekaran, 1987; Grubb and Mawson, 1993; Dale, *et al.*, 1994; Silveira, *et al.*, 2005; Zafar, *et al.*, 2005; Ozmihci and Kargi, 2007a, b, c). These problems have been commonly attributed to osmotic sensitivity (due to the high lactose concentrations) and low ethanol tolerance (Janssens, *et al.*, 1983; Vienne and von Stockar, 1985; Grubb and Mawson, 1993; Zafar, *et al.*, 2005; Guimarães, *et al.*, 2010).

Data presented in Table (3) show the effect of the different concentrations of lactose on the growth of the four selected yeast strains; namely Z-2, Z-5, Z-13 and Z-39.

Table (3). Effect of different lactose concentrations on the growth of selected yeasts.

Strains	Lactose 2%	Lactose 10%	Lactose 40%	Lactose 60%
Z-2	+++	++	+	+
Z-5	+++	++	+	+
Z-13	+++	++	+	+
Z-39	++	-	-	-

+++ Normal growth   ++ Moderate growth
+ Faint growth   - No growth

Ozmihci and Kargi (2007b) used pure culture of *Kluyveromyces marxianus* (DSMZ 7239) to ferment Cheese whey powder (CWP) solution with different CWP or sugar concentrations to ethanol in a continuous fermenter.

RAPD analysis

RAPD – analysis was performed to determine the variation at the molecular level among the four strains.

A total of five random tenmer primers (OPA-2, OPA-3, OPA-9, OPA-11, and OPA-15) were used in the current investigation and the variations were determined through the RAPD profiles.

Each of these primers generated between 9 and 20 bands, ranging from 149 to 1704 bp. All the oligonucleotides tested gave four different patterns, indicating that they are suitable for strain discrimination. According to Mitrakul, *et al.*, (1999) and Martorell, *et al.*, (2005), OPA-2, OPA-3 and OPA-9 were the best primers to discriminate yeast strains. On the other hand, Table (4) illustrates also that the total number of bands was of 85, whereas the total number of polymorphic bands was of 82 and the polymorphism percentage was 96%.

The results of RAPD – analysis obtained by primers OPA-2, OPA-3 and OPA-9 are illustrated in Figure (2) and by primers OPA-11 and OPA-15 are in Figure (3).

The description of each primer generated bands are presented in the following:

Figure (2): Agarose gel electrophoresis of RAPD products by OPA-2, OPA-3 and OPA-9 primers.

Primer (OPA – 2)

Data in Figure (2) illustrate results of RAPD-analysis obtained by this primer, which contains 80% (G + C). The number of amplified fragments generated by this primer was 19, which ranged in size between 149 and 1367 bp. Table (4) shows that the polymorphic percentage was 100%.

Primer (OPA-3)

Data in Figure (2) illustrate results of RAPD-analysis obtained by this primer. This primer contains 60% (G + C), it reacted with all isolates. The total num-ber of amplified fragments which was generated by this primer was 20. The size of these bands varied among the isolates from 179 to 1334 bp. Table (4) shows that the polymorphic percentage was 95%.

Primer (OPA-9)

The total number of bands generated by this primer which contains 70% (G + C) was 19 which ranged in size between 223 to 1704 bp as shown in Figure (2). Table (4) shows that the polymorphic percentage was 89 %.

Figure (3): Agarose gel electrophoresis of RAPD products by OPA-11 and OPA-15 primers.

Primer (OPA – 11)

RAPD – analysis using the OPA–11 primer are illustrated in Figure (3). This primer contains 60 % (G + C) and produced18 fragments, which ranged in size between 258 to 1601 bp. Table (4) shows that polymorphic percentage was 100 %.

Primer (OPA – 15)

Figure (3) shows the results of RAPD analysis obtained from OPA-15 primer had also 60 % (G + C), it reacted with all four isolates generating 9 fragments ranging in size between 227 and 1243 bp. The number of generated bands varied among isolates from 13 to 19. Table (4) illustrates that the percentage of polymorphism was 100 %.

Table (4) Polymorphism obtained by RAPD analysis among different isolates

primer	Total number of bands (a)	Number of polymorphic bands(b)	Polymorphism -b/a * 100%
OPA-2	19	19	100
OPA-3	20	19	95
OPA-9	19	17	89
OPA-11	18	18	100
OPA-15	9	9	100
Total	85	82	96

Genetic similarity matrix and cluster analysis

Data of the presence/absence of DNA fragments phenotypically analyzed using MVSP program of (Nie and Li, 1979), and pair-wise comparisons between the tested isolates

of yeasts were used to calculate the genetic similarity. Then, based on the calculated genetic similarity presented in Table (5), an estimation of the relationship between different isolates was concluded. Data showed also that the lowest genetic similarity was observed between isolate Z-2 and Z-13, Z-5 and Z-39, Z-13 and Z-39 (0%), while the highest value was found between Z-5 and Z-13 (46.1538%) belonging to the same genus.

Table (5): Genetic similarity values calculated from the DNA fragments amplified from the different isolates of yeasts using five RAPD primers.

	Z-2	Z-5	Z-13	Z-39
Z-2	1.000000			
Z-5	0.153846	1.000000		
Z-13	0.000000	0.461538	1.000000	
Z-39	0.363636	0.000000	0.000000	1.000000

The four isolates of yeasts were regrouped in two clusters based on the genetic similarity given in Table (5), and dendrogram as in Figure (4). The first group included the two isolates Z-13 and Z-2, while the second group was represented by the other two isolates, Z-39 and Z-2.
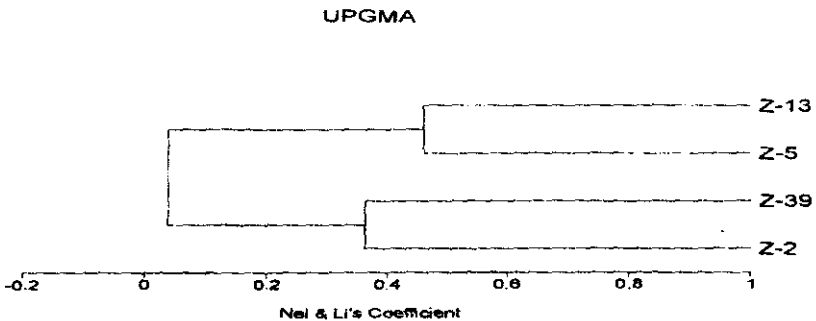
UPGMA

Figure (4): Dendrogram demonstrating the relationship among four isolates of wild yeasts based on data recorded from polymorphism of RAPD markers.

Couto, *et al.*, (1994 and 1995) indicated that in RAPD fingerprint, the level of differentiation, either inter-or intraspecies, depend highly on the primers used. Moreever Sineo, *et al.*, (1993) indicated that RAPD/PCR analysis can give important information if analyzed for linkage studies, gene mapping or phylogenetic purposes.

The application of RAPD analysis, by using genomic DNA from yeast strains belonging to the genera *Saccharomyces, Zygosaccharomyces and Kluyveromyces*

using 10-mer oligonucleotides, proved to be a powerful tool for yeast discrimination (Paffetti, *et al.*, 1995; Andrighetto, *et al.*, 2000). Lathar, *et al.*, (2010) reported that the genetic diversity of yeasts isolated from seventeen different fruit can be studied by using RAPD analysis.

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283

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دراسات وراثية جزيئية على الخمائر الطبيعية المعزولة من المنتجات اللبنية المصرية

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يعتبر شرش الجبن ناتج مصاحب للصناعات اللبنية، ينتج بكميات هائلة وله عبء تلوثى عالى، لذلك فهو يمثل مشكلة بيئية خطيرة على مستوى العالم. واللاكتـــوز الموجود فى شرش الجبن يمكن أن يستغل كمادة تفاعل لإنتاج الإيثانول الحيـوى وذلك عن طريق التخمر الميكروبى حيث أن هضمه اللاهوائى يقدم وسيلة ممتازة لكل من تخزين الطاقة والتحكم فى التلوث بالإضـــافة الـــى أنـــه مـــن ارخـص مصادرالكربون المستخدمة لإنتاج الإيثانول. وقد استهدفت الدراسة الحالية الكشف عن سلالات خميرة قادرة على تخمير اللاكتوز الموجود فى شرش الجبن، والتـــى تم عزلها من عينات أمكن جمعها من مناطق جغرافية مختلفة فى مصر. تم عزل 39 عزلة خميرة والتى تم انتخاب أربع سلالات منها والتى اشير اليها بـــالرموز (ز-2، ز-5، ز-13، ز-39) وذلك على اساس نـــشاطها التخميـــرى وتحملهـــا للاكتوز، واظهرت النتائج أن السلالات ز-2، ز-5، ز-13 نمت بشكل طبيعـــى على تركيزات 2% و 10% من اللاكتوز، ثم اتبعها قـــدرة أقـــل للنمـــو علـــى التركيزات الأعلى حتى نسبة 60%. وعلى الجانب الآخـر فـان العزلـة ز-39 أظهرت نمو متوسط على تركيز 2% فى حين لم تتمكن من النمو على التركيزات الأعلى الأخرى . وقد تم تعريف هذه السلالات الاربع على المستوى الجزيئـــى: (ز-2) تتبع الجنس بيشيا ، (ز-5) ، (ز-13) تتبع الجنس كلوفيرومايسيس أمـــا (ز-39) فإنها لاتتبع جنس الــ سكاروميسيس ، كما تمت دراسة العلاقة الوراثيـــة فيما بين هذه السلالات المنتخبة بإستخدام خمسة بادئات مختلفة واظهرت النتـــائج لبصمة الـ DNA أنماط حزمية مميزة للسلالات الأربع المدروسة. وقد وضعت فى مجموعتين، اشتملت الأولى على السلالتين ز-5، ز-13 فـــى حـــين ضـــمت الثانية ز-2، ز-39.