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Determination of Enzyme Profiles and Molecular Characterization of Yeast Species Isolated from Butter Samples

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Abstract

Constituting the most important group of microorganisms that offered to human consumption through food, yeasts are a substantial part of microflora in dairy products. Due to sophisticated enzyme production especially high proteolytic and lipolytic activities, some of the yeast species play an essential role in many processes such as fermentation, ripening, production of aroma precursors and deterioration in dairy product indicating these products as a source of extracellular enzyme producer yeast strains. In this study, butter samples were collected from delicatessens and grocery stores in different locations in İzmir province in order to isolate enzyme producers. A total of 40 yeast strains were isolated and investigated for their lipase, protease and amylase activities on solid medium consisting of tributyrin, skim milk and starch, respectively. Sixteen yeast isolates were found to be positive for enzyme production. Lipase and protease activities were observed in 11 yeast strains (68,75%), while only one yeast strain (6,25%) was found to be positive for protease and amylase production. 4 of yeast strains (25%) were able to produce only lipase enzyme. Molecular characterization of the yeast strains based on 26S rRNA region D1/D2 domains revealed that the isolates belonged to *Candida zeylanoides* (62,5%) and *Pichia fermentas* (37,5%) species.

Keywords — Amylase, identification, isolation, lipase, yeast, protease, 26S rRNA sequencing

1. Introduction

Industrial enzymes play key roles in the production of innumerable biotechnology products in different fields ranging from cleaning supplies, food and beverages to pharmaceuticals [1, 2, 3]. These enzymes can be found from different sources including plants, animals and microorganisms. Enzymes from microbial origins, which constitute more than 50% of industrial enzymes, are much preffered as it's easy to cultivate the microbial cells within inexpensive media and scale up [4]. Even they catalize the same reaction, different microorganisms synthesize disparate enzymes that provides some flexibilities regarding the optimization of conditions for a specific production process [5]. Following the selection of appropriate enzymes, production level can be induced by 100-fold through the recombinant DNA techniques easily applied. Additional genetic manipulations, like mutagenesis, will further improve enzymatic properties in terms of thermostability, optimal pH, substrate specificity and other kinetic parameters [6].

In various industrial processes, microbial enzymes have several advantages over chemical catalysts such as ability to function under mild conditions of pH and temperature, selective catalysis of the reaction without any byproduct(s), less energy consumption, nontoxic to nature owing to their complete biodegradability, etc [1, 3]. Total market for industrial enzymes is segmented into three sections; (i) technical enzymes, (ii) food & beverage enzymes and (iii) other enzymes including chemicals and pharmaceuticals (Figure 1). Among them, technical enzymes, which generally used as bulk enzymes to reduce the production cost, are in the first place with $2.2 billion estimated value at 2016 [4]. These enzymes includes proteases, lipases, amylases, cellulases and many others in detergent, textile, biofuel, pulp and paper, food, as well as dairy industries. In particular, proteases and lipases are of special interest in dairy industry since their activities are substantial in coagulation of milk for cheese production and flavor development. However,

lipolytic and proteolytic activities in milk products may sometimes cause unwanted odor and tastes such as rancid or soapy tastes if an uncontrolled lipolysis and proteolysis occurs. Since the high nutritional profile and low pH, dairy products provide a favorable environment for spoilage yeasts [7, 8].

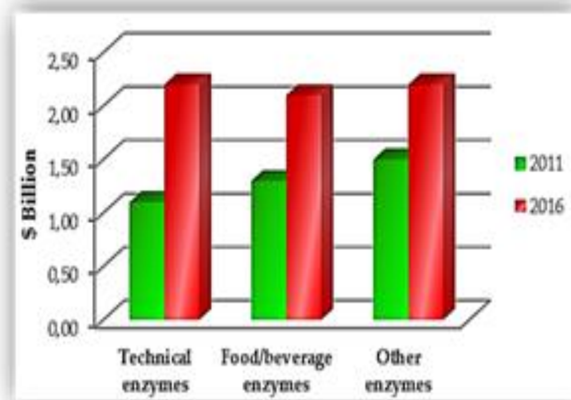


Figure 1. Global industrial enzyme market values between 2011 and 2016 [9]

As a rapid growing sector of biotechnology, enzyme industry is always searching for the new enzymes most of which are hydrolytic in action fulfilling the requirements of a specific industrial process [10]. Considering their biodiversity, fast growth rate and susceptibility to genetic manipulation, microorganisms serve as the most important enzyme source [2, 11]. Thus, in the paper, it was aimed to isolate and identify enzyme producer yeasts from various butter samples.

2. Material and Methods

2.1 Sample collection and isolation of yeast strains

The butter samples used in this study were obtained from various markets and delicatessens of İzmir province. Samples were transferred to the laboratory under aseptic conditions and subjected to isolation procedure to obtain enzyme producer yeast strains. The butter samples were initially homogenized and diluted in sterile peptone water. Using pour plate technique, one mL from each dilution was taken in a sterile petri dish and approximately 20 mL of sterile Yeast extract Peptone Glucose agar (YPG, g/L: yeast extract, 10; peptone, 20; glucose, 20; agar, 20) was poured into the plates. After incubation at 27°C for 3 days, colonies with different colony morphology were picked up and pure cultures were obtained by streaking several times on YPG agar plates. The pure yeast isolates were maintained on YPG agar plates at 4°C by reviving every two months.

2.2 Determination of lipolytic, proteolytic and amylolytic activity on agar plate

Enzyme activities for lipase and protease were tested on Potato Dextrose Agar (PDA, g/L: potato extract, 4; dextrose, 20; agar, 15) plates supplemented with tributyrin (1%) and skim milk (10%), respectively. To determine amylase activity, Amylase Activity Medium (AAM, g/L: peptone, 5; yeast extract, 5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; NaCl, 0.01; soluble starch, 5; agar, 15) was used. Cell suspension of fresh cultures of yeast strains were prepared in sterile peptone water (0,1%) and spotted on above mentioned agar plates. After the incubation at 27°C for 3 days, the hydrolysis zone around the yeast colonies and colony diameter were measured and scored. For visualization of amylase activity, starch plates were flooded with Gram's iodine solutions (1% KI and 0.1% I_2) [12]. Enzymatic activity was scored as '+++' when the diameter of hydrolysis zone (R) is bigger than 5 mm, '++' when the diameter of hydrolysis zone is between 3-5 mm, '+' when the diameter of hydrolysis zone is smaller than 3 mm, and '-' when no hydrolysis zone was observed.

2.3 Genomic DNA isolation of the yeast strains

DNA extraction was performed according to the method described by Liu et al. [13]. Overnight cultures of pure isolates grown in YPG broth were transferred to eppendorf tubes and centrifuged at 7000 g for 5 min at 4°C to harvest the cells. 500 µL of lysis buffer (400 mM Tris-HCl, pH 8.0; 60 mM EDTA, pH 8.0; 150 mM NaCl; 1% SDS) was added to the pellet and the tube was vortexed until a homogeneous mixture was obtained. The mixture was incubated at 65°C for 10 min and vortexed again after the addition of 150 µL of potassium acetate (pH 4.8; which is made of 60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid, and 28.5 mL of distilled water). The mixture was centrifuged at 12.000 g and 4°C for 5 min and the supernatant was transferred into a sterile tube. Genomic DNA in aqueous phase was precipitated with equal volume of isopropanol and then washed with 70% cold ethanol. The DNA was dried at room temperature, subsequently dissolved in nuclease-free water and stored at -20°C until use.

2.4 Sequencing of D1/D2 domain of 26S rRNA region

For amplification of D1/D2 domain of 26S rRNA, PCR reactions were performed using primer pair of NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3'). Amplification of the target regions were completed under the following conditions; 5 minutes of initial denaturation at 95°C, 40 PCR cycles consisting of 1 minute of denaturation at 95°C, 2 minutes of annealing at 58°C and 3 minutes of extension at 72°C, with final elongation for 10 minutes. Sequencing analysis of PCR products were done using ABI 3130XL automated sequencer (Applied Biosystems, USA).

2.5 Phylogenetic analysis of molecularly characterized yeast strains

The sequences obtained from a previous step were compared pairwise using BLASTN search tool and by using multiple alignment software CLUSTAL W version 2.0. These sequences were aligned with the sequences of related species retrieved from GenBank. Tamura-Nei neighbor joining method was used for the construction of phylogenetic tree by MEGA software version 7.0 [14, 15]. Confidence levels of the clades were estimated from bootstrap analysis (1,000 replicates) [16].

3. Results and Discussions

In various studies, it was reported that butter samples provide heterogeneous distribution of yeast populations and can be used for yeast isolation [17, 18]. Also, several authors showed that numerous yeast strains in dairy products hydrolyze milk fat through their lipolytic enzymes they produce [18, 19]. In our study, butter samples were investigated for yeast isolation, especially strains having enzyme production. A total of 40 yeasts isolated from the samples were screened for their lipolytic, proteolytic and amylolytic activity (Figure 2). Although broad range of variation was observed in the activities, only sixteen isolates exhibited a hydrolysis zone against at least one substrate. As seen in Table 1, 68,75% of the isolates hydrolyzed tributyrin and skim milk at different levels. On the other hand, 4 out of 40 yeast isolates (25%) produced only lipase enzyme while only one yeast (6,25%) was found to be positive for protease and amylase enzymes.

Figure 2. Detection of lipase (a), protease (b) and amylase (c) activities of the yeast isolates on solid medium

The isolates showing enzymatic properties were characterized on the basis of sequence analysis of D1/D2 domain of 26S rRNA region. PCR amplification of the region with primers NL1 and NL4 rendered products varying in sizes from 438 bp to 574 bp (Table 2). After sequencing of these products, the sequences were compared with GenBank database using the BLASTN search and accession numbers were reserved. Results revealed the presence of two species belonging to two different genera (Table 2). Ten isolates were identified as *Candida zeylanoides* while six isolates were *Pichia fermentans*. These results are in agreement with those of previous researches which showed that *Candida* and *Pichia* strains isolated from dairy products have the capacity to produce relevant enzymes [17, 18, 20].

In Figure 3, phylogenetic analysis of the D1/D2 domain of the 26S rRNA sequences of the yeast strains isolated from butter samples by the neighbor joining method was presented. Related and out-group species including *Candida zeylanoides* CBS947, *Galactomyces candidum* CBS357.86, *Saccharomyces cerevisiae* CBS6237, *Pichia fermentans* CBS1876 and *Yarrowia lipolytica* CBS7133 were retrieved from GenBank database to determine the placement of the isolated strains in the phylogenetic tree.

Table 1. Enzyme activities of the yeast isolates

Isolate no	Hydrolysis zone (R)		
	Lipase	Protease	Amylase
TEMH4	++	+++	–
TEMH6	–	+++	+
TEMH8	++	++	–
TEMH10	++	+	–
TEMH27	+++	–	–
TEMH29	++	++	–
TEMH31	+	++	–
TEMH32	++	+	–
TEMH33	++	++	–
TEMH38	++	++	–
TEMH40	+++	+	–
TEMH41	++	+	–
TEMH53	+++	–	–
TEMH59	++	+	–
TEMH61	+++	–	–
TEMH65	+++	–	–

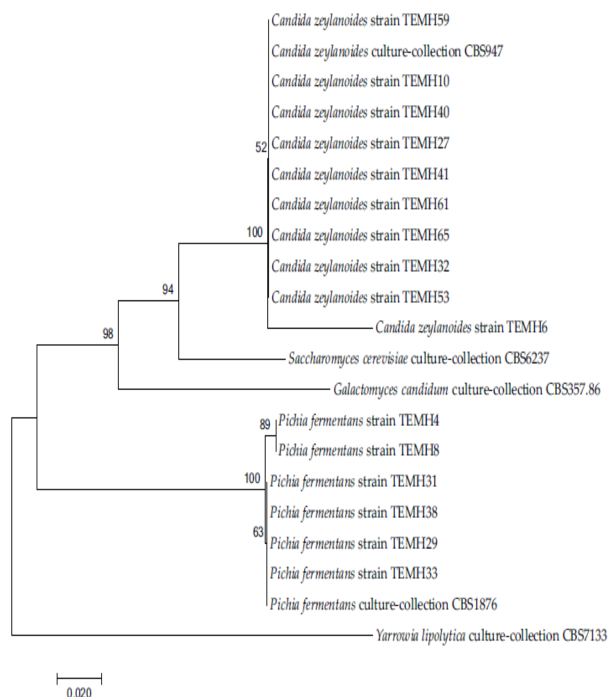
-: no activity, +: R<3 mm, ++: 3≤R<5 mm, +++: R≥5 mm

Table 2. Molecular identification results of the yeast isolates

Isolate no	Sequencing of D1/D2 domain of 26S rRNA			Species name
	26S rRNA PCR (bp)	Homology (%)	Accession number	
TEMH4	557	99	KJ413160.1	*Pichia fermentans*
TEMH6	438	96	KJ413161.1	*Candida zeylanoides*
TEMH8	563	99	KJ413162.1	*Pichia fermentans*
TEMH10	574	99	KJ413163.1	*Candida zeylanoides*

TEMH27	571	99	KJ413164.1	*Candida zeylanoides*
TEMH29	563	99	KJ413165.1	*Pichia fermentans*
TEMH31	549	99	KJ413166.1	*Pichia fermentans*
TEMH32	566	100	KJ413167.1	*Candida zeylanoides*
TEMH33	563	99	KJ413168.1	*Pichia fermentans*
TEMH38	558	99	KJ413169.1	*Pichia fermentans*
TEMH40	573	99	KJ413170.1	*Candida zeylanoides*
TEMH41	568	99	KJ413171.1	*Candida zeylanoides*
TEMH53	560	99	KJ413172.1	*Candida zeylanoides*
TEMH59	574	99	KJ413173.1	*Candida zeylanoides*
TEMH61	567	99	KJ413174.1	*Candida zeylanoides*
TEMH65	567	99	KJ413175.1	*Candida zeylanoides*

Figure 3. The placement of yeasts in phylogenetic tree constructed by the neighbor joining method based on the large subunit rRNA.



4. Conclusions

Microbial enzymes are obtained from various sources to catalyze the reactions consisting of chemo-, enantio-, and regio-selective transformations. As a result of these specificities, the target products of enzymatic reactions need less purification and refining procedures that reduces overall cost. The industrial demands for new enzyme sources with novel enzymatic properties and low costs have promoted the isolation of new enzyme producers. Most of these extracellular enzymes have been derived from various microbial sources. From the industrial viewpoint, the yeasts isolated from butter samples in our research have the ability of being a source for industrial processes. No doubt, further researches are required for the use of these strains and their products in the industrial applications.

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References

1. Illanes, A, Cauerhff, A, Wilson, L, Castro, G.R, Recent trends in biocatalysis engineering, *Bioresource Technology*, 2012, 115, 48-57.
2. Adrio, J.L, Demain, A.L, Microbial enzymes: Tools for biotechnological processes, *Biomolecules*, 2014, 4(1), 117-139.
3. Choi, J.M, Han, S.S, Kim, H.S, Industrial applications of enzyme biocatalysis: current status and future aspect, *Biotechnology Advances*, 2015, 33(7), 1443-1454.
4. Prakash, D., Nawani, N., Prakash, M., Bodas, M., Mandal, A., Khetmalas, M., Kapadnis, B, Actinomycetes: A repertory of green catalysts with a potential revenue resource, *BioMed Research International*, vol. 2013.
5. Sanchez, S., Demain, A.L, Enzymes and bioconversions of industrial, pharmaceutical, and biotechnological significance, *Organic Process Research & Development*, 2011, 15(1), 224-230.
6. Singhania, R.R, Patel, A.K, Pandy, A, The industrial production of enzymes. In: Soetaert W, Vandamme E.J (eds) Industrial Biotechnology: Sustainable Growth and Economic Success, John Wiley & Sons, Germany, 2010, pp 207-225.
7. Champagne, C.P, Laing, R.R, Roy, D, Mafu, A.A, Griffiths, M.W, Psychrotrophs in dairy products: their effects and their control, *Critical Reviews in Food Science & Nutrition*, 1994, 34, 1-30.
8. Ledenbach, L.H, Marshall R.T, Microbiological spoilage of dairy products. In: Sperber WH, Doyle MP (eds) Compendium of the Microbiological Spoilage of Foods and Beverages, Springer, New York, 2009, pp 41-67.
9. Global markets for enzymes in industrial applications.. http://www.bccresearch.com/pressroom/bio/global-market-industrial-enzymes-reach-nearly-%247.1-billion-2018, 2014 (accessed 26.01.2017).
10. Kirk, O, Borchert, T. V, Fuglsang, C. C, Industrial enzyme applications, *Current Opinion in Biotechnology*, 2002, 13(4), 345-351.
11. Demain, A.L, Adrio, J.L, Contributions of microorganisms to industrial biology, *Molecular Biotechnology*, 2008, 38(1), 41-45.
12. Fossi, B.T, Tavea, F, Jiwoua, C, Ndjouenke, R, Screening and phenotypic characterization of thermostable amylases producing yeasts and bacteria strains from some Cameroonian soils, *African Journal of Microbiology Research*, 2009, 3(9), 504-514.

13. Liu, D, Coloe, S, Baird, R, Pedersen J, Rapid mini-preparation of fungal DNA for PCR, *Journal of Clinical Microbiology*, 2000, 38(1), 471.

14. Tamura, K, Nei, M, Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees, *Molecular Biology and Evolution*, 1993, 10(3), 512-526

15. Kumar, S, Stecher, G, Tamura, K, MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets, *Molecular Biology and Evolution*, 2016, 33(7), 1870-1874.

16. Limtong, S, Kaewwichian, R, Jindamorakot, S, Yongmanitchai, W, Nakase, T, *Candida wangnamkhiaoensis* sp. nov., an anamorphic yeast species in the Hyphopichia clade isolated in Thailand, *Antonie van Leeuwenhoek*, 2012; 102(1), 23-28.

17. Lopandic, K, Zelger, S, Banszky, L.K, Eliskases-Lechner, F, Prillinger, H, Identification of yeasts associated with milk products using traditional and molecular techniques, *Food Microbiology*, 2006; 23(4), 341-350.

18. Sagdic, O, Ozturk, I, Bayram, O, Kesmen, Z, Yilmaz, M.T, Characterization of butter spoiling yeasts and their inhibition by some spices, *Journal of Food Science*, 2010, 75(9), M597-603.

19. Fleet, G.H, Mian, M.A, The occurrence and growth of yeasts in dairy products. *International Journal of Food Microbiology*, 1987, 4(2), 145-55.

20. Corbo, M.R, Lanciotti, R, Albenzio, M, Sinigaglia, M, Occurrence and characterization of yeasts isolated from milks and dairy products of Apulia region, *International Journal of Food Microbiology*, 2001, 69(1), 147-152.