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Isolation and Characterization of Starter Culture from Spontaneous Fermentation of Sourdough

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ABSTRACT

Wheat flour dough samples were collected from different bakeries located in Faisalabad city, Pakistan. Spontaneous fermentation of dough samples was carried out for 24 h at 30°C in the laboratory. The isolates were Gram stained and the Gram positive were identified to genus level using morphological, physiological tests. Identification based on classical identification methods and API 50 CH assimilation profiles showed that the lactobacilli contained in sourdoughs belonged to three groups (*Lactobacillus brevis, L. plantarum & L. fermentum*). Yeasts were also characterized on the basis of morphological and biochemical criteria. Conventional identification methods and API 20 C AUX assimilation profiles revealed that *Saccharomyces cerevisiae* was the only yeast species present on the sourdoughs.

Key Words: Sourdough; API 50 CH; API 20 C AUX; Lactobacilli; Yeast

INTRODUCTION

Bacterial starters have been produced for a variety of fermented products to improve their sensory and other quality characteristics. Spontaneous fermentation has been used for the production of fermented foods based on the microflora present in the raw material (Vogel *et al*., 2002). The quality of end-product was dependent on the types and number of microorganism in the raw material. Spontaneous fermentation was optimized through back slopping i.e., inoculation of the raw material with a small quantity of a previously performed successful fermentation, which means dominance of the best adapted strains (Harris, 1998). The direct addition of selected starter cultures to raw materials is a milestone in the production of fermented foods, which may help control the overall standardization of the fermentation process and quality of the end product. Strains with the specific physiological and metabolic properties were isolated from natural habitats or from successfully fermented products for use in the industrial productions (Oberman & Libudzisz, 1998).

Lactic acid bacteria (LAB) are a group of Gram-positive bacteria, catalase-negative, non-motile, non-sporeforming rods or cocci and produce lactic acid as the major end product during the fermentation. They are strictly fermentative, microaerofile, acidophilic, salt-tolerant with complex nutritional requirements for carbohydrates, amino acids, peptides, fatty acids, salts, nucleic acids derivatives and vitamins. The natural habitat of these organisms includes humans, animals and plants. Their long history of safe use (Holzapfel *et al.*, 2001), commonly referred to as the GRAS (Generally Recognized As Safe) status, has led to a wide range of industrial applications i.e., flavor, texture and preservative qualities of many fermented foods such as cheese, yoghurt, sausages, sourdough breads. Several species belonging to the genera *Leuconostoc, Weissella, Pediococcus, Lactococcus, Enterococcus and Streptococcus* have been isolated from sourdough, but *Lactobacillus* strains are the most abundant. The overall flavor of the bread is affected by the content of lactic and acetic acid. Homofermentative LAB are able to convert hexoses almost completely into lactic acid (>85%), whereas heterofermentative LAB degrade hexoses into lactic acid, acetic acid/ethanol and CO_2. The temperature also affects the ratio of lactic acid to acetic acid in addition to type of starter culture. Lactic and acetic acids are also produced by heterofermentative LAB from pentoses (Hammes & Vogel, 1995). Yeasts are also present in sourdoughs such as *S. exiguous, Torulopsis holmii, Candida krusei, Pichia norvegensis* and *Hansenula anomala* but *S. cerevisiae* is frequently present or added (Gobbetti *et al*., 1994).

The starter cultures for the production of fermented products are not presently produced in Pakistan and are imported for industrial use. Mostly yeasted preferments are being used for the production of white bread. The use of LAB as starter culture may help to improve the quality and shelf life of the products. The LAB of the naturally fermented sourdoughs may be used in the production of

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novel fermented foods such as sourdough bread, which is likely to have superior quality and longer shelf life. Therefore, the present study was designed to characterize the suitable starter culture for the production of sourdough bread.

MATERIALS AND METHODS

Sample collection. Fifteen sourdough samples were collected from different bakeries in Faisalabad, Pakistan using sterilized jars. The samples were kept at 4–8°C and transported in special cool boxes within hours to the Institute of Food Science and Technology, University of Agriculture, Faisalabad, Pakistan for analysis.

Viable count. A 10 g of sourdough sample was homogenized with 90 mL of sterile diluent (5 g peptone, 8.5 g NaCl, 1000 mL distilled water, pH 7.0±0.2). Further decimal dilutions were prepared with the same diluent. Total aerobic mesophilic bacteria were enumerated on plate count agar (PCA, Oxoid, Basingstoke, Hampshire, UK) (Lonner *et al*., 1986). MRS (deMan Rogosa Sharpe) agar (Oxoid, UK) was used for total LAB counts (Man *et al*., 1960). A 10 mg L^{-1} of cycloheximide (Merck, Darmstadt, Germany) was added to the media for prevention of the growth of yeasts and moulds. Acidified potato dextrose agar (PDA, Oxoid, UK), pH 3.7, was used for enumeration of yeasts (Okada *et al*., 1992).

Isolation and identification of lactobacilli. Presumptive lactobacilli were selected from higher dilution nutrient agar plates and tested for cell morphology, Gram reaction and catalase formation by adding 3% H_2O_2 directly onto each plate. The isolates were purified by successive streaking on the Rogosa agar and MRS agar media before characterization. The agar plates were incubated anaerobically (BBL Gas Pak, H_2 & CO_2; Becton-dickinson, Cockeysville, MD, USA) for 24 h at 30°C for the isolation of lactobacilli. The Gram positive and catalase negative strains were subjected to the following physiological and biochemical tests: gas (CO_2) formation from glucose, arginine hydrolysis, growth in 2%, 4% and 6.5% NaCl, growth at 5, 15 and 45°C (Harrigan & McCance, 1990). The fermentation pattern among carbohydrates was determined by using the API 50 CH gallery with the API 50 CHL medium (Biomerieux, France). Anaerobiosis in the inoculated tubes was obtained by overlaying with sterile paraffine oil. The inoculated galleries were incubated at 30°C and the observations were made after 24 and 48 h. The identification of the isolates was facilitated with the use of a computer program, APILAB PLUS, version 3.2.2. (Biomerieux) and reference to Bergey's Manual of Systematic Bacteriology (Wood & Holzapfel, 1995). The pure bacterial isolates were inoculated into MRS broth, incubated for 24 h at 30°C, centrifuged (Sigma, 3K30, Germany) at 3000 rpm for 15 min and the supernatant was decanted. The cell pellets were resuspended in sterile MRS broth containing 10% (v/v) glycerol. The suspension was aseptically transferred into sterile cryotubes containing acid-washed glass beads and stored at -80°C (De Man *et al*., 1960).

Isolation and identification of yeasts. Yeasts were isolated by plating on PDA acidified to pH 3.5. Plates were incubated for 3 days at 25°C and then yeast colonies were counted. Representative colonies were isolated and purified by streak plating using the same medium. The yeast isolates were subjected to various morphological and physiological tests, which included urease test, growth at 37°C, presence of pseudohyphae using yeast extract malt extract media, growth and size of vegetative cells in a liquid medium by direct microscopic examination. Identification was made with reference to standard descriptions (Harrigan & McCance, 1990; Beuchat, 1993; Deak, 1993). Yeast isolates were further characterized for their assimilation patterns using API 20 C AUX gallery (Biomerieux). The purified cultures were routinely maintained on PDA slants and kept at 4°C.

RESULTS AND DISCUSSION

Number of microorganisms. The microbiological composition of the laboratory fermented sourdough samples revealed that the total aerobic mesophilic bacteria (TAMB) count ranged between 7.07×10^5 to 6.89×10^9, the lactic acid bacteria (LAB) 6.24×10^4 to 6.92×10^7, the yeast 6.35×10^3 to 7.95×10^7 and coliform 3.47×10^1 to 1.67×10^4 (Table I). The results obtained in the present study are in close agreement with those reported for sourdoughs by Gobbetti (1998) and Zinedine (2007).

The sourdoughs are ecosystems, where fundamental interactions between LAB and yeasts take place. The predominant organisms are LAB containing significant numbers of yeast cells (Vogel *et al*., 1999). LAB are mainly responsible for the acidification of the sourdough, whereas the sourdough yeasts are very important for the production of flavor compounds and for a well balanced flavor in combination with the acids. The LAB may either originate from natural flour contaminant, a fermented dairy product or from a commercial starter culture containing characterized strains of LAB produced through batch fermentation (Vuyst & Neysens, 2005). These results suggested that the sourdoughs tested in the study were contaminated with LAB and yeasts. Moreover, most of the samples contained LAB and yeast cells within the range reported by Vuyst and Neysens (2005).

Composition of lactobacilli and yeast. The presumptive lactobacilli were randomly isolated from the MRS agar plates and divided into three groups based on their several morphological and physiological characters. Lactobacilli showed a characteristic cell morphology appearing as a combination of thin, short and long rods in pairs or short chains. The colonies on MRS agar were irregular, white and rough sometime possessing a raised centre. Similar findings have also been reported by Zahoor (2005) who isolated

Table I. Microbiological characteristics of laboratory fermented sourdoughs (CFU g^{-1})

Sourdough samples	TAMB	LAB	Yeast	Coliform
S_1	7.07×10^5	6.24×10^4	6.35×10^3	1.22×10^2
S_2	6.36×10^6	5.67×10^6	7.64×10^4	3.47×10
S_3	8.14×10^5	7.08×10^5	7.83×10^4	2.90×10^3
S_4	8.03×10^7	7.15×10^6	8.41×10^3	6.35×10
S_5	9.25×10^5	8.36×10^5	8.01×10^3	1.06×10^3
S_6	7.72×10^6	6.20×10^6	5.27×10^7	1.15×10^2
S_7	5.88×10^6	5.03×10^5	7.95×10^4	2.44×10^3
S_8	7.61×10^8	7.00×10^6	6.44×10^5	2.09×10^1
S_9	6.77×10^7	5.41×10^6	6.35×10^7	4.53×10^2
S_{10}	8.44×10^8	5.67×10^6	7.64×10^6	1.67×10^4
S_{11}	8.25×10^7	7.08×10^5	7.83×10^6	3.87×10^3
S_{12}	6.89×10^9	6.92×10^7	8.41×10^6	1.58×10^2
S_{13}	7.64×10^7	6.22×10^6	8.01×10^5	2.39×10^2
S_{14}	9.01×10^7	6.05×10^6	5.27×10^7	2.95×10^2
S_{15}	7.55×10^7	5.03×10^7	7.95×10^7	3.62×10^1

CFU = Colony forming units
TAMB = Total aerobic mesophillic bacteria
LAB = Lactic acid bacteria

Table II. Characterization of lactobacilli isolates from sourdoughs

Group	I	II		III	
Subgroup	A	B	C	D	E
No. of isolates	17	13	7	9	6
Cell shape	Rods	Rods	Rods	Rods	Rods
Gram staining	+	+	+	+	+
Catalase reaction	-	-	-	-	-
CO_2 from glucose	+	+	-	-	+
NH_3 from arginine	+	80(10)	-	-	+
Growth at 5°C	-	-	-	-	-
Growth at 15°C	75(25)	+	+	+	-
Growth at 45°C	-	-	-	-	+
Growth in 2% NaCl	+	+	+	+	+
Growth in 4% NaCl	35(60)	-	+	+	+
Growth in 6.5% NaCl	-	-	-	-	45

Symbols: +, positive reaction; -, negative reaction; d, weak reaction; 80(10), 80% strains positive, 10% strains weakly positive

LAB from commercial curd samples. Within lactobacilli, group I and II, which contained the largest number of isolates showed growth at 15°C and not at 45°C but differed for CO_2 production from glucose and NH_3 production from arginine. The group III did not show growth at 15°C but grew at 45°C and produced CO_2 from glucose and NH_3 from arginine (Table II). The presumptive isolates belonging to the above groups were further characterized using the API 50 CHL system. Sugar fermentation profiles in the API 50 CH (Table III) indicated that the isolates could be assigned to three groups corresponding to individual species. In general, all the strains fermented glucose, fructose, maltose, ribose and gluconate. None of these fermented erythritol, D-arabinose, L-xylose, adonitol, β-methyl-xyloside, L-sorbose, dulcitol, inositol, starch, glycogen, xylitol, D-xylose, D-tagatose, D-fucose, L-fucose, L-arabitol and 2-keto-gluconate (Table III).

According to the species description of Spicher and Schroder (1978), Kandler and Weiss (1986) and Hammes and Vogel (1995), 46% of the isolates were identified as *Lactobacillus brevis* (belonging to group I), 24% as *L. plantarum* (belonging to group II) and 8% as *L. fermentum* (belonging to group III). The present findings are in line with those of Gobbetti *et al.* (1994) who isolated *L. brevis* (49%), *L. plantarum* (21%) and smaller percentages of *L. fermentu*m with an overall percentage of heterofermentative isolates of 58% as compared to 54% found in our study. The results of the present study are also in concordance with the several Italian (Gobbetti *et al*., 1994; Ottogalli *et al.,* 1996), German (Spicher, 1987) and Spanish (Barber *et al*., 1983) sourdoughs showing the associations of hetero and homofermentative strains. The results of the present study are in contrary to the findings of Corsetti *et al.* (2001) who reported that the sourdoughs consisted of either only one species or a very complex association of several species belonging to only one metabolic class. Similarly, Wood and Holzapfel (1995) reported that the *L. brevis* and *L. fermentum* were the dominating microorganisms in fermenting plant material and sourdoughs. The differences might be due to variation of the species present in the raw material used in both studies.

Table III. Fermentation of carbohydrates by the isolates tested by API 50 CH gallery

Group	I	II		III	
Sub group	A	B	C	D	E
L-arabinose	+	+	+	-	d
Cellobiose	-	-	+	+	+
Esculin	-	-	+	+	-
Galactose	+	+	+	+	+
Lactose	-	-	+	+	+
D-mannose	-	-	+	+	-
Melizitoze	-	-	+	+	-
Melibiose	+	+	+	+	+
D-raffinose	68	+	+	+	+
Saccharose	+	+	+	+	+
Trehalose	-	82	+	+	-
D-xylose	+	74	-	-	36
Rhamnose	-	-	62	d	-
Manitol	-	-	+	+	-
Sorbitol	-	-	+	+	-
Glycerol	-	-	-	-	-
Amygdaline	-	-	+	+	-
Salicin	-	-	+	+	-
Arbutin	+	+	+	+	-
Inulin	-	-	-	-	-
a-Methyl-D-glucoside	+	+	+	+	-
N-Acetyl glucosamine	+	+	+	+	-
β-Gentibiose	-	-	+	+	-
D-Turanose	-	-	+	+	-
D-Arabitol	-	-	+	+	-
5-keto-gluconate	+	+	-	-	-

API = Analytical Profile Index
Symbols: +, positive reaction; -, negative reaction; d, weak reaction; 80, 80% strains positive

After a preliminary morphological screening, the yeast isolates were selected and examined through the API 20 C AUX system. The results indicated that isolates corresponded to S. *cerevisiae,* which were in agreement with several taxonomic studies (Spicher, 1983; Gobbetti *et al.*, 1994; Gobbetti, 1998). A large number of *S. cerevisiae* isolates is certainly related to the addition of baker's yeast to the dough.

CONCLUSION

The spontaneously fermented sourdough can be used as a source for the isolation of starter cultures (*L. brevis, L. fermentum, L. plantarum*) and for the production of several fermented products. The sourdoughs contained both homo and heterofermentative lactobacilli in coexistence, which have great potential to impart the particular characteristics in the final product through fermentation. The benefits of sourdough incorporation in the bread production demand that sourdough technology should be used on commercial scale production of bakery products to improve their quality and shelf life.

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