Selection of mixed starters for the preparation of traditional Moroccan bread

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Abstract

The main objective of this work was the selection of mixed starters with a combination of Lactic Acid Bacteria (LAB) and yeast strains for traditional bread production in Morocco. For this, a total of 21 LAB strains and 36 yeast strains were isolated from different traditional sourdough. Dough fermentation were assessed by monitoring physicochemical parameters including, titratable acidity, decrease of pH and lactic acid, ethanol and CO_2 production. A total of six yeasts and four LAB were selected for their technological performances. Morphological, physiological and biochemical identification performed using API identification kits confirmed that these strains belonged to *Saccharomyes cerevisiae, Candida humilis* and *Saccharomyces exiguus* species, and *Lactobacillus plantarum* and *Lactobacillus casei* species for yeasts and LAB respectively. The yeast S3-L2 and the LAB OD-BL5 strains exhibited the best performances among the selected ones; S3-L2 yeast strain were able to produce ΔV=23mL of CO_2 and showed the highest values of ethanol and biomass production (2.87 g/L and 1.25 10^9 UFC/mL, respectively). Whereas OD-BL5 LAB strains produced 13.9 g/L of lactate in dough. These findings lead to consider these two strains very good candidates for the formulation of an effective mixed starter for bread preparation. Subsequently, sensorial analysis results showed that bread prepared using mixed starter No. 24 composed of the two selected species exhibited better exterior appearance, golden and crispy crust, large volume and honeycomb crumb, compared to the control.

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Introduction

Bread is believed to be the most complete and cheap food and basic auxiliary food in times of extreme food poverty.[1,2] Bread is often fermented before baking by authorized fermentation agents, used simultaneously or not, such as baker's yeast and sourdough. The importance of bread in human diet, especially in the Mediterranean area, has been recognized since Greek times.[1]

Sourdough is a mixture of flour and water, fermented with lactic acid bacteria and yeasts, which can grow spontaneously or be inoculated as selected ferments.[3] It is characterized by a higher number of Lactic Acid Bacteria (LAB) than yeasts. Many studies have shown that the use of sourdoughs rather than pure yeast results in breads of higher nutritional value and which can be stored for a longer period of time.[4] Natural sourdoughs are composed mainly of flour, water, and microbial communities composed of a dominant species of LAB and a dominant species of yeast, and at most a few dozen minority species. The knowledge acquired through microbial ecology suggests that the biological diversity of sourdoughs is an asset for its stability to face changes in methods, practices and/or the invasion of microbial species.[5] This knowledge also shows that there is a relationship between species diversity and the functioning of ecosystems. Beyond the production of CO_2, organic acids and ethanol by fermentation, yeasts and LAB produce aromatic compounds such as alcohols,

aldehydes, diacetyl, acetoin and esters[6] and decrease the negative effect of certain metabolites such as phytic acid.[7] In addition, these microorganisms contribute to lowering the glycemic index and improving the texture and flavor.[8]

Sourdoughs can be obtained from different methods, either by spontaneous fermentation or using selected starters. According to the technological parameters applied, the sourdoughs are divided into three groups according to the predominant fermentation consortia:[9] type I sourdoughs are fermented between 20 and 30 °C and the two main organisms are *Lactobacillus sanfranciscensis* and *L. pontis*. Type II use baker's yeast as a leavening agent and the dominant lactic acid bacteria strains are *L. pontis*, *L. panis* and one to nine other lactobacilli. Type III are dried products of traditional fermentation which are dominated by lactic acid bacteria resistant to drying, such as *Pediococcus pentosaceus, L. plantarum* and *L. brevis*. Other organisms found in some sourdough fermentations include *Candida humilis*, *Dekkera bruxellensis*, *Saccharomyces cerevisiae*, and *Saccharomyces uvarum*.[9-11]

Physicochemical parameters, such as pH, Total Titratable Acidity (TTA), water activity (a_w), humidity, ash and protein, and microbiological count (viability of lactic acid bacteria and yeasts) are used to evaluate the performances of starters.[12]

In Morocco, bread is a staple food eaten one to three times a day. Commercial bakeries only feed a portion of the population with industrial bread, while the bulk of households bake their own bread using either commercial leaven or a traditional sourdough starter which is produced and stored by back-slopping.[13] Improving the fermentative performance of commercial baker's yeasts remains one of the most important biotechnology challenges.[14]

Despite the industrial performance demonstrated for commercial yeast, the bread prepared by it starts to be singled out for its nutritional properties and its potential effects on consumer health. Indeed, the unhydrolyzed form of phytic acid combines with minerals in the digestive tract, especially calcium and magnesium (but also iron, zinc and copper), which leads to demineralization by lack of organic assimilation.[15] However, slow fermentation process occurred in the case of sourdoughs, and thanks to bacterial phytases it led to a rapid and total decrease in the content of phytic acid.[16] The acidification and enzymatic catalysis carried out by LAB breaks down most of the phytic acid into inositol (vitamin B) and bio-available calcium and magnesium phosphates. On the other hand, the artisanal production of sourdough and sourdough bread still suffers from several problems: a multitude of methods for obtaining the sourdoughs, instability and difficulty in storing, excessive acidity of the bread produced, long operational times and non-reproducible results, etc.[17]

The main objective of this study is to design an efficient and operational mixed starter for the production of good quality traditional bread. Combinations of selected microorganisms were studied and used for the preparation of high performance stabilized mixed starters. The results of this study should lead to develop a process for the large-scale production of typical traditional Moroccan bread in a reproducible and controlled manner while retaining all the nutritional and therapeutic virtues of dietetic bread.

Materials and Methods

Samples

Nine samples of traditional sourdough recovered from the central region of Morocco (Skhirate-Temara), the South-East (Errachidia) and the North (Tetouan) were used as a source for isolation of yeast and LAB strains. Samples were collected in sterile containers and stored aseptically at 4°C until use. Table 1 shows the codes assigned for each of the sourdoughs samples as well as the ingredients used in their preparation.

Isolation, purification and identification of yeast and LAB strains

Enrichment was carried out in liquid Yeast Peptone Glucose (YPD) selective medium[18] and de Man, Rogosa and Sharpe (MRS) medium,[19] respectively for yeast and LAB. Afterwards, 0.5 mL of each medium was inoculated in plates containing YPD and MRS agar media, respectively for yeasts and LAB using pour-plate method. The plates were incubated at 30°C for 24–48h. A succession of 3 subcultures were carried out in order to purify the strains. Morphological and physiological characterization of isolates was performed by microscopic examination, Gram stain, catalase and oxidase activity as well as gas production.[20,21] Biochemical identification of the isolated yeast and LAB strains was performed using API 20C AUX[22] and API 50 CHL[20] identification kits. The carbohydrate fermentation profile has been appropriately determined and characterized according to the manufacturer's instructions (Biomerieux, France). The pure strains were then coded and immersed in a 40% glycerol solution in 2mL cryotubes and stored at -20 °C for further utilization.

Table 1. Samples of sourdoughs collected.

Sample code	Region	Age of sourdough	Ingredients used in the preparation of sourdough
S1	Mers El Khair	1 week	Couscous+flour+whey
S2	Mers El Khair	1-2 weeks	Flour+water+couscous
S3	Sidi Betache	2 years	Flour+water
S4	Mers El Khair	1 month	Flour+sourwhey
OD	Errachidia	2weeks	Date and barley
FB	Temara	1 week	Broad bean and soft wheat
BL	Temara	1 week	Wheat and whey
OL	Temara	1 week	Barley and whey
LT1	Tetouan	1 month	Durumwheatflour+whey

Potential of selected strains

CO_2 production in dough by yeast

CO_2 production is one of the most important criteria used to judge the performance of yeast, since it affects the texture of the bread crumbs after baking. To test the ability of the selected yeast strains to produce CO_2, 20g portions of dough, prepared from a commercial soft wheat flour (Kenz), were inoculated with a 2mL suspension (10^6 CFU/mL) of each selected yeast strain. Then, the portions of dough were placed in 100 mL test tubes so as to fill a volume of approximately 20mL. The test tubes were sealed with parafilm and incubated at 30°C for 12 h. The variation in volume (ΔV) was measured at the end of incubation. An unseeded test tube served as a control.[23,24] The commercial *Saccharomyces cerevisiae* strain (Rafiaa) was used as a reference.

pH monitoring in dough

Having a low pH in dough and sourdough inhibits growth of pathogenic bacteria. The pH of dough was determined using an Adwa-AD1000 pH meter (Hungary). The pH-electrode was punched in 10% (w/v) slurries of dough disaggregated in 100mL of distilled water.[25]

Ethanol production

The concentration of produced ethanol by each strain of yeast was determined using High Performance Liquid Chromatography (HPLC). For this, the strains were cultured in 500 mL YPD medium at 30 °C for 48 hours. Then, culture samples were centrifuged at 13,000 rpm for 10 min and the supernatants were filtered through a 0.2µm filter to remove remaining cells. The filtrates were then stored in 2mL tubes containing 0.5% sodium azide.[26] The ethanol analysis was carried out using a YOUNG-Lin HPLC equipped with a Supelcogel C-610H column (30cm x 7.8mm; column heated to 40 °C) and RID detector. The detection cell was maintained at 35 °C. An isocratic mobile phase composed of 0.1% H_3PO_4 (prepared in MilliQ water) was used at a flow rate of 0.8 mL/min. The duration of the analysis was set at 35 min operating at a maximum pressure of 60 bars.[27] Commercial *Saccharomyces cerevisiae* strain was used as a reference.

Cellular biomass

The cell biomass of yeast and LAB strains was monitored using a spectrophotometer at a wavelength of 650nm.[28] A preliminary correlation between the actual cell concentration determined by Malassez cell count and that estimated by absorbance measurement was made.

Titratable acidity and lactic acid production by LAB

The capacity of LAB to acidify the dough was studied. The titratable acidity expressed in g of lactic acid/100mL was measured by titration with a 0.1N NaOH solution using phenolphthalein as a color indicator. In addition, the selected LAB strains were tested for their ability to produce lactic acid by conducting fermentations in 500mL flasks containing modified MRS medium (supplemented with 20g/L lactose). The lactate produced was then determined by HPLC following the same protocol mentioned above.

Bread-making tests using the mixed starters

Combinations of mixed starters were formulated by associating the best performing yeast and LAB strains. CO_2 production and titratable acidity were then monitored. The mixed starter cultures were prepared from precultures carried out in the modified MRS and YPD media respectively for the yeasts and the LAB. Based on the performance tests, the best performing mixed starter was selected to be used in a bread-making test on commercial soft wheat flour using the following proportions: 500g flour, 9g salt, 10g pate starter, 300 mL water.[29] The bread-making steps are described in Table 2. The bread produced by the efficient mixed starter was compared with the bread produced by commercial yeast under the same conditions. The qualitative parameters, external appearance, crust, crumb, volume and texture, were evaluated.

Results

Isolation

A total of 57 strains of yeast and lactic acid bacteria were isolated using specific medium. The results of the morphological and physiological characterization allowed to identify 36 strains of yeast and 21 strains of lactic acid bacteria (Table 3).

Table 2. Steps followed for bread making.

Processingstep	Time
Kneading	t_0
Fermentation-First flap	t_0 +1h10min
Fermentation-Second flap	t_0 +2h12min
Shaping - thirdflap	t_0 +3h
Putting in the oven (30min of baking)	t_0 +4h
Out of the oven	t_0 +4h33min

Table 3. Codes of isolated and purified Lactic Acid Bacteria (LAB) and yeasts.

Sourdoughs[a]	Yeasts strains code[b]	LAB strains code[b]	Number of isolated LAB	Number of isolated yeasts
FB	FB-Ln	FB-BLn	5	0
OD	OD-Ln	OD-BLn	7	1
OL	OL-Ln	OL-BLn	10	2
S1	S1-Ln	S1-BLn	5	6
S2	S2-Ln	S2-BLn	4	6
S3	S3-Ln	S3-BLn	6	6

[a]Acronyms representing the types of sourdoughs depending on the ingredients used in their preparation (Table 1); [b]L, Yeast; BL, Lactic Acid Bacteria; n, increment number.

Phenotypic identification of the isolated yeast and LAB strains was performed using API 20C AUX and 50 CHL identification kits, respectively. The results indicated that the isolates corresponded to *Saccharomyes cerevisiae* (14 strains), *Saccharomyces exiguus* (7 strains), *Candida humilis* (5 Strains), *Candida krusei* (4 strains), *Debaryomyces hansenii* (4 strains), and *Saccharomyces uvarum* (2 strains), whereas the identified LAB strains through API Web platform were *Lactobacillus casei* (5 strains), *Pediococcus pentosaceus* (4 strains), *Lactobacillus fermentum* (4 strains), *Lactobacillus plantarum* (3 strains), *Lactobacillus brevis* (2 strains), *Lactobacillus alimentarius* (1 strain), *Leuconostoc mesenteroides* (1 strain), and *Lactobacillus sanfranciscensis* (1 strain).

Performance tests of yeast

CO_2 production and dough rise

The fermentative activity of the selected yeast strains was determined by measuring their capacity to produce carbon dioxide. For this test, a control (non-inoculated) was used each time for each strain tested to eliminate the effect of the starting microbial load. A subtraction was made between the CO_2 value of the tested strain and the control. Figure 1, shows the results of the CO_2 volumes produced in the test tubes according to the different yeast strains tested.

Figure 1 shows that the best CO_2 production was noted for the S1-L3 strain isolated from S1 sourdough. For this strain, a ΔV of 25mL was produced from a 20g portion of dough compared to the commercial reference strain *S. cerevisiae* (noted S.C) (ΔV=16 mL). S3-L2 and S3-L3 were also able to produce a notable CO_2 volume (ΔV=23 mL), besides FB-L1 and S3-L6 (ΔV=22 mL), S3-L1 (ΔV=21 mL), S3-L4 (ΔV=20 mL), FB-L3 and OL-L1 (ΔV=19 mL), respectively. The other strains tested remained less successful in terms of CO_2 gas production capacity. Some strains presented a ΔV close to zero which means that they did not show significant fermentative activity.

Ethanol production and cell biomass

Figure 2 highlights the values of ethanol concentrations (expressed in ppm) for the different strains tested. The commercial strain *S. cerevisiae* (denoted S.C) was used as a reference.

According to the results depicted in Figure 2, the values of biomass and ethanol revealed good correlation. Besides, the OL-L4, OL-L5, OL-L9 and OL-L10 strains isolated from the OL sourdough were able to produce fairly high concentrations of ethanol (1625.82 ppm; 1684.09 ppm; 1656.40 ppm; 1657.03 ppm, respectively). On the other hand, the OD sourdough strains revealed low values in both biomass and produced ethanol (10.95 to 381.48 ppm). On the other side, we could note that for the FB sourdough strains, the FB-L3 isolate took the highest value of ethanol production (1383.99 ppm) followed by the FB2 isolate (1101.88 ppm). These two strains were among the yeasts that were able to produce significant biomass.

Performance tests carried out on LAB

The selected LAB were tested for their ability to acidify the MRS medium and for their potential to produce lactic acid which is an essential component in the mixed sourdough fermentation process.

Acidity and pH control

The S3-BL2 and OL-BL1 strains produced the highest concentrations in this series with an acidity of 0.23% and 0.22% (w/w), respectively. The S3-BL4 strains could produce 0.20% of lactate in dough. The S3-BL1, S2-BL5, S1-BL5 and S3-BL5 strains produced a similar acidity of around 0.18%. On the other hand, the pH was monitored before and after fermentation that lasted 5h. The S2-BL4, S2-BL5, S2-BL6 and S1-BL2 strains exhibited the same pH after fermentation which was 4.1. The only strains that could respect the optimal value (4.3) were S3-BL3 and OD-BL5. The S1-BL1, S3-BL4 and OL-BL1 strains showed low pH values compared to S1-BL3, S1-BL5, S1-BL6, S3-BL1, S3-BL2, S3-BL5 and S-BL6 strains which revealed high pH values.

Production of lactic acid (lactate)

Figure 3 highlits the results of the amounts of lactic acid produced by LAB strains determined by HPLC. The S2-BL6 and S2-BL5 LAB strains from the S2 sourdough, OL-BL1 from the OL sourdough and OD-BL5 from the OD sourdough were able to produce the highest concentrations of lactic acid which were 14562;

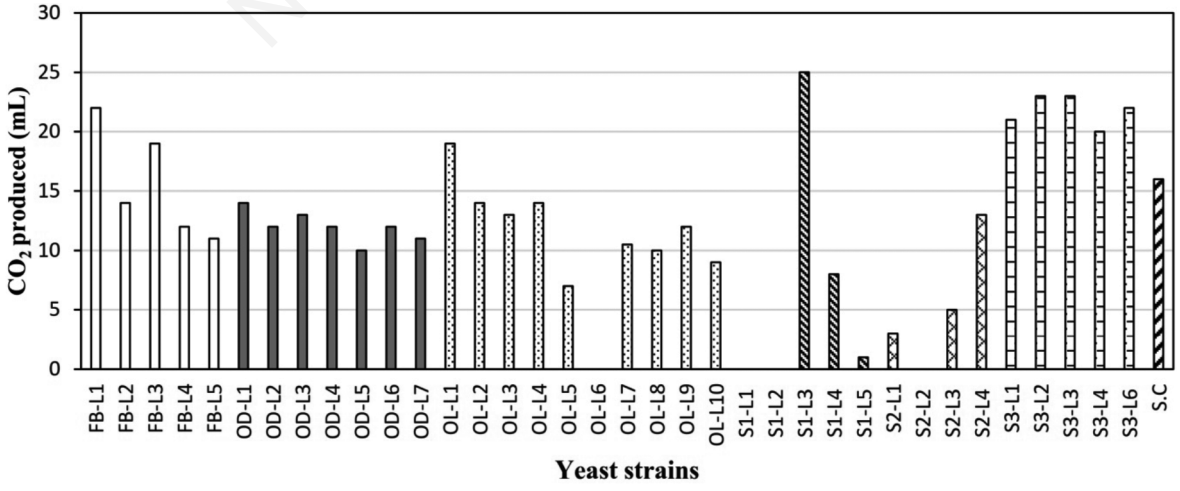

Figure 1. CO_2 produced by different yeast isolates. The values of the CO_2 volume produced represent the differences (ΔV) respectively between the volumes measured in the inoculated samples and in the control.

7862; 10933; 13916 ppm respectively. The lowest value, which is 163 ppm, was produced by OL-BL2 strain from OL sourdough. The other strains could not exceed 4000 ppm.

Mixed starters test

A variety of combinations of highly efficient strains were performed. The six yeast strains selected were namely S3-L2, OL-L4, OL-L5 and OL-L9 belonging to *Saccharomyes cerevisiae* species, and OL-L10 and B-L3 belonging to *Candida humilis* and *Saccharomyces exiguus* species. Therefore, the choice was made on 4 high-performant LAB which are S2-BL2, S2-BL6, and OD-BL5 (*Lactobacillus plantarum*) and OL-BL1 (*Lactobacillus casei*).

CO_2 production

CO_2 produced by the mixed starters was determined using the same protocol given above. The results are displayed in Figure 4. From the Figure 4, the highest value (ΔV=19.5 mL) of CO_2 was exhibited by starter 9, followed by starter 17 and 25 which generated a ΔV of 18.5 and 16.5 mL of carbon dioxide, respectively.

Starters 1, 10, 11, 13, 16, 20, 22 and 24 were shown to have almost the same ability in CO_2 production, they were able to produce an average volume of ΔV of 14.08 mL.

Starters 7 and 21 were shown to produce the lowest volumes in carbon dioxide which were 1 and 2.5 mL, respectively. The other starters produced CO_2 between 5 and 10 mL.

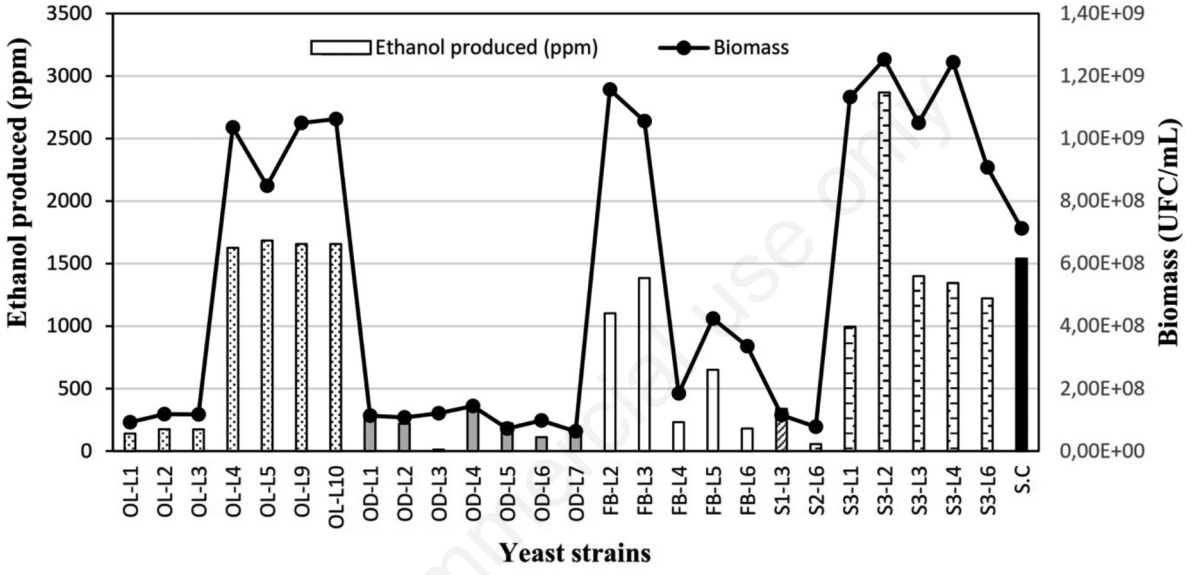

Figure 2. Ethanol and cell biomass concentrations obtained for the selected yeast strains.

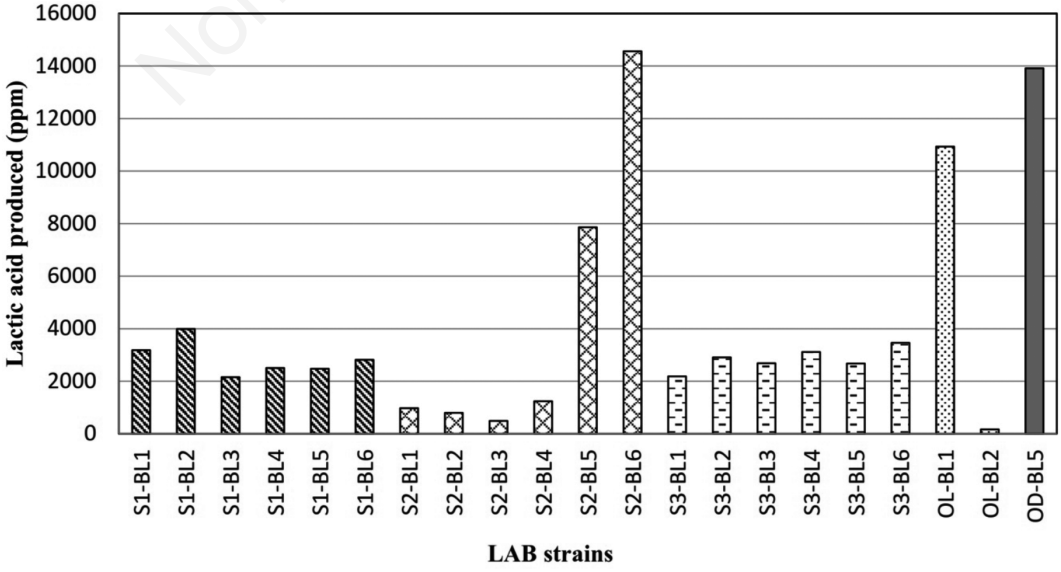

Figure 3. Concentrations of lactate expressed in ppm, produced by the different isolated strains of lactic acid bacteria.

Acidity and pH

So far, few systematic studies have been conducted on the effects of common organic acids such as acetic acid, lactic acid and malic acid on the textural characteristics and sensory qualities of bread. However, organic acids, which can act as acidifying and antioxidant agents, have been traditionally added to foods for thousands of years.[30]

According to Figure 4, starters 18, 19, 20, 22, 23, and 24 exhibited lactate production of above 4g/L. Starter 7 was able to produce 3.675g/L lactic acid, whereas starters 9, 10, 13 and 29 showed the lowest values. The remaining starters were able to produce concentrations ranging from 1.1to 2.7g/L which are not sufficient for bread making.

Concerning the pH monitoring, the initial values of the pH of the various mixed starters did not exceed 6 at time t_0. At the end of the fermentations, starters 9;13;17;21 and 29 have kept a neutral pH. Contrary, the rest of the starters showed pH which are slightly acidic with values lower than 5. The most acidic mixed starters were 7; 11; 14 and 15 which have a pH value below 4. These results differ from those obtained by Chaoui *et al.*.[23] Their study indicates pH values of an average of 3.09, lower than the European standard that sets a pH 4.3 in the dough of good quality.[31]

Bread-making test

The selection of the efficient mixed starter was done according to variance analysis and Student Newman Keuls test using SAS software.

Two types of breads were produced by mixing the ingredients with different microbial starters. The first one was prepared using the commercial yeast *Saccharomyces cerevisiae* and the second one using the selected starter 24 consisting of the yeast strain S3-L2 and the LAB strain OD-BL5.The organoleptic characteristics of the two breads were then assessed. The obtained result showed that the bread made with the commercial yeast strain has a normal odor and a light brown color. The texture of the crumb is not very porous, the shape is flat and the crust is thin, this is due to the low production of gas (CO_2) in this bread. On the other hand, the bread produced from the starter 24 has a fruity smell thanks to the production of organic acids. The color was dark brown. Besides, the alveolar texture, the domed shape and the thick crust refer to the production of gas which is the criteria of performance used for the choice of the starter 24.

Furthermore, the storage life of the two breads was studied. The quality of the bread made with baker's yeast was maintained for a period of 4 days at room temperature (27°C). After this period, the bread became inedible. Contrary, the bread made using starter 24 has shown stable quality during 7 days under the same temperature (27°C).

Discussion

Isolation

From the nine traditional sourdoughs studied, 57 strains of yeast and lactic acid bacteria were isolated using specific medium. The identified strains have been reported in previous publications to be species characterizing bread-making sourdough. For example, Meroth *et al.*[32] reported that they isolated *Candida humilis*, *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, and *Saccharomyces uvarum* from German traditional sourdoughs produced under practical conditions. *Lactobacillus sanfranciscensis*, *L. plantarum*, *L. paralimentarius*, *L. fermentum*, *L. casei*, and *Pediococcus pentosaceus* were isolated by Kitahara *et al.*[33] from different japanese sourdoughs. However, according to De Vuyst *et al.*,[34] typical sourdough yeast species are *Candida humilis*, *Kazachstania exigua*, and *Saccharomyces cerevisiae* and typical sourdough LAB species are *Lactobacillus fermentum*, *L. paralimentarius*, *L. plantarum*, and *L. sanfranciscensis*.

Performance tests of yeast

The fermentative activity of the selected yeast strains was determined by measuring their capacity to produce carbon dioxide, as this parameter reflects the leavening capacity of the dough.[35] The method used determined the amount of CO_2 released into the headspace, which remains a suitable method although it overlooks the amount retained in the dough.[36]

From Figure 1, S1-L3 strain exhibited the best CO_2 production

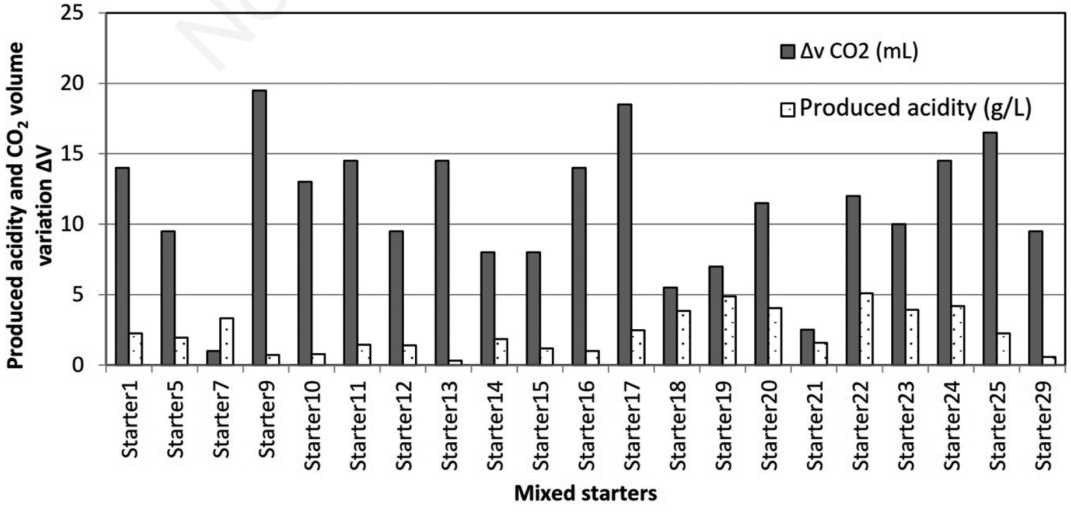

Figure 4. Acidity and CO_2 produced according to the different mixed starters. The CO_2 volume values produced represent the respective differences (ΔV) between the volumes measured in the inoculated samples and the one in the control.

(ΔV=25 mL) among the 36 yeast strains tested. In a similar study, the results obtained by Codină *et al*.[37] revealed higher dough rise values than ours with values of 58.50, 65.50 and 57.50 mL respectively at incubation times of 60, 120 and 180 min but using a rheofermentometer. The rheofermentometer was used to maintain automatically a stable pressure (1.25 kg) throughout all the fermentation period. In addition, the flour used in this study was mixed with 3% yeast and 1.5% salt and the dough was kneaded for 15 min and then fermented under controlled conditions of temperature and relative humidity. Consequently, the low gas production values obtained in our study were probably due to the non-control of the pressure in the tubes (the gas pressure in the tubes increases as it is produced). As an outcome of this test, the strains that showed a significant CO_2 production ability were selected for subsequent investigations.

The bioconversion capacity of fermentable sugars into ethanol was assessed for the selected yeast strains. In addition, the ethanol concentration in the YPD culture medium was determined by HPLC. On the other hand, the biomass was determined measuring the absorbance at 650 nm in order to investigate if there is a significant correlation between the cell biomass and the ethanol produced. From results depicted in Figure 2, FB-L3 followed by FB2 isolates were among the yeasts that were able to produce significant biomass. However, the representative strains of sourdough S3 showed good performance both in terms of ethanol production and biomass. Indeed, the S3-L2 strain showed the highest values for the two parameters studied (2868.74 ppm and 1.25×10^9 respectively for ethanol and biomass), whereas strains 4 to 10 of the OL sourdough were the only strains that showed better performances than those exhibited by the reference strain. In the other side, the S1-L3 strain revealed slightly lower values for the two performance parameters studied, despite the good performance of this strain in terms of dough leavening.

Performance tests of LAB

In addition to the role of lactic acid as a taste and aroma enhancer of sourdough bread, it has been reported that it contributes also in acidifying the substrate and thereby inhibits the growth of pathogens and undesirable agents causing organoleptic changes.[38] The optimal concentration of lactic acid reported is 0.4% (w/w).[30] Acidity was determined by titration using 0.1N NaOH. The obtained results showed that the S3-BL2 and OL-BL1 strains produced the highest values of titratable acidity of 0.23% and 0.22% (w/w), respectively. In addition, these two strains showed pH values around the optimal pH value of sourdough bread which is 4.3 according to Lhomme *et al.*[31] On the other hand, lactate production capacity have been carried out in order to identify performant LAB strains wich can catalyse efficient lactic fermentation. Indeed, lactate is one of the desired products in a traditional sourdough bread.[31] The results showed that except OL-BL2, all the LAB strains were able to produce concentrations of lactate above 900 ppm, which is the minimum desired value in bread according to Lhomme *et al.*[31]

Mixed starters and bread-making test

After completing the performance tests on the different isolated strains, the best performing strains were selected to produce functional mixed starters. The performance tests (CO_2 and acidity productions) were performed on the different combinations of produced starters. The best performing mixed starters were selected for a bread making test in which the quality of the breads produced was determined according to organoleptic and physical characteristics. The development of starter cultures in artisanal plants and the application of good hygienic practices (to avoid contamination) represent a valuable approach to control and optimize fermentation conditions, and thus contribute significantly to the improvement of these traditional technologies.[39] This allows to control and accelerate the fermentation process by working only with specific strains with regard to bioconversion objectives, thus, leading to achieve good yields.

The six yeast strains selected to prepare the mixed starters were namely S3-L2, OL-L4, OL-L5 and OL-L9. The production of carbon dioxide is a criterion for the selection of efficient starters. In fact, a desirable bread volume is only achieved if the dough provides a favorable environment for yeast growth and gas production and, at the same time, has a gluten matrix capable of maximum gas retention.[40] From the Figure 4, the highest value of CO_2 (ΔV=19.5 mL) was exhibited by starter 9, followed by starter 17 and 25. The results obtained by Chaoui *et al.* showed lower values than those obtained by this study, while using a large portion of dough around 300g.[23] On the other hand, starters 18, 19, 20, 22, 23, and 24 could exhibited lactate production of above 4 g/L which are conceivable concentrations to produce a traditional bread.[30] Faid *et al.*[13] used a 10 g portion to measure acidity by titration. Their results showed high lactate production (around 0.75%) compared to the results of the current study. This value exceeds the standard set in bread which is 0.4% of dough.[30] This is probably due to the incubation time, which was 6h, exceeding 5h used for the starters in this study, showing that one hour can increase the production of acids in the dough. The results reported by Hadaegh *et al.*[41] showed a high acidity of around 1% (w/w) for the SD3BY mixed starter. While the lowest acidity was produced by the SDT5Y starter, namely 0.53% (w/w), which far exceeds the lowest acidity produced in the present study.

The reference flour for obtaining good quality leavened bread is derived from wheat because of its innate and unique protein fractions composed of glutenin and gliadin.[42] The selection of the efficient mixed starter for bread making was done according to variance analysis and Student Newman Keuls test using SAS software. Based on organoleptic characteristics, the bread produced from the starter 24 was better appreciated by the evaluators compared to the reference bread produced by commercial yeast. Furthermore, the bread made using starter 24 has shown stable quality during 7 days under 27°C compared to 4 days for the control. This is most probably due to the organic acids produced by the mixed starter 24.

Conclusions

Traditional bread is a prized food in Morocco because of its cultural and nutritional values. When properly processed, natural sourdough produces more digestible bread than the conventional one. Thus, the use of sourdough in the bread-making process is an effective way to fight against the demineralization caused by complexing phytates.[15] Indeed, bacterial phytases produced by LAB leads to a rapid transformation of most of the phytic acid into inositol (vitamin B) and bio- available calcium and magnesium phosphates.[16]

The main objective of this study was to formulate efficient mixed starter composed of yeast and LAB strains isolated from natural sourdough in Morocco in order to produce traditional bread of high quality. For this purpose, samples of natural sourdoughs of different compositions have been collected from several housewives in Morocco.

In order to select the best performing strains, CO_2 and ethanol production, and lactic acid production have been monitored respectively for yeasts and LAB selected strains. Consequently, six yeasts coded S3-L2, OL-L4, OL-L5, OL-L9, OL-L10 and B-L3 and four

LAB coded (S2-BL2, S2-BL6, OD-BL5 and OL-BL1) were selected for their technological performances. The yeast S3-L2 and the LAB OD-BL5 strains exhibited the best performances among the selected ones; S3-L2 yeast strain were able to produce ΔV=23 mL of CO_2 and showed the highest values of ethanol and biomass production (2868.74ppm and 1.25×10^9 UFC/mL, respectively). Whereas OD-BL5 LAB strains produced 13.9 g/L of lactate in dough. These findings lead to consider these two strains very good candidates for the formulation of an effective mixed starter for bread preparation. This could be explained by the values of CO_2 and lactates (produced respectively by the yeast and LAB strains) considered as main parameters reflecting the leavening capacity of the dough.[35] Therefore, these two strains were used to formulate starter 24.

Finally, the results of bread making test allowed to state that the bread produced with the selected mixed starter 24 has a better organoleptic quality than the one prepared with commercial *Saccharomyces cerevisiae*. Thus, the results of this study should lead to develop a process for the large-scale production of typical traditional Moroccan bread in a reproducible and controlled way while retaining all the nutritional and therapeutic virtues of dietetic bread. However, future studies should test the possibility to cost effectively produce ready-to-use high-performant stabilized mixed starters from endogenous strains by means of preservation techniques like drying or freeze-drying.

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