INTRODUCTION

In bread-making, fermentation by yeasts is of primary importance for its leavening function and the possible contribution to the production of desirable flavour compounds. Although the baking industry generally uses strains of *Saccharomyces cerevisiae,* in rural areas in the north of Portugal a special type of corn and rye bread is still prepared using dough carried over from a previous making as a starter. This piece of dough is usually kept in cool places, covered with a layer of salt. Prior to bread-making, it is mixed with fresh flour and water and, when fully developed, serves as the inoculum for the bread dough. This starter dough is, likely, a natural biological system characterized by the presence of yeasts and lactic acid bacteria living in complex associations in a system somewhat similar to that existing in sour doughs. While the microflora of sour dough and the basis of the sour dough process are well known (Kline *et al.* 1970; Sugihara *et al.* 1970, 1971; Kline and Sugihara 1971), in the case of traditional corn and rye breads the information is virtually non-existent including possible interactions between yeasts and other micro-organisms, particularly lactic acid bacteria. With this perspective, the present work describes the isolation of yeasts from home-made corn and rye bread doughs collected at different locations and the characterization of the yeast populations. The yeasts were identified and further characterized for the occurrence of killer activity. Growth rates and biomass values in different media were also determined in order to select for strains of potential value for the baking industry.

Correspondence to: Dr Célia Santos Pais, Departamento de Biologia, Universidade do Minho, Campus de Gualtar, 4709 Braga Codex, Portugal.

MATERIALS AND METHODS

Isolation and identification of the yeasts

Thirty-three dough samples collected from farms mainly located in the north of Portugal were examined. The pH of the dough samples was determined electrometrically using a pH meter model 91 WTW (Wissenschaftich Technische Werkstatten). A loopful of dough (±1 g) was resuspended in 20 ml of sterile water and submitted to vigorous shaking until completely mixed. For yeast isolation, 0·3 ml of the suspension obtained was directly inoculated onto YM Agar (Difco) with glucose (1% w/v) and agar (2%), supplemented with chloramphenicol (500 mg l^{-1}) to inhibit bacterial growth. For the quantitative analysis of the yeast population in each dough, further dilutions of the dough suspension were made before spreading onto plates containing 2% (w/v) glucose, 1% (w/v) bacto-peptone, 0·5% (w/v) bacto-yeast extract and 2% (w/v) agar. The plates were incubated at room temperature until yeast colonies could distinctly be observed and their morphologies compared. Colonies were selected and isolated on the basis of their different macro and micromorphological characteristics, and proportionally to their frequency of occurrence. The yeasts were then purified by streaking on medium with the same composition without supplements. After purification the yeasts were maintained on 2% malt extract agar slopes at 4°C. Isolation of yeasts from commercially available baker's yeast was also performed for comparisons. The identification of the isolates followed the standard methods described by van der Walt and Yarrow (1984).

Detection of killer activity

All the yeast isolates were tested for their ability to kill selected strains as indicated in Table 2. Sensitive strains were supplied by the Portuguese Yeast Culture Collection (PYCC), Gulbenkian Institute of Science, Oeiras. Yeast strains to be tested for killer activity were inoculated, in concentrated zones (±1 cm diam.), onto plates containing YM medium with methylene blue (30 mg l^{-1}) and adjusted to pH 4 with 0·1 mol l^{-1} citrate buffer. Those plates had been previously spread with a lawn of sensitive strain, grown in YM medium (van der Walt and Yarrow 1984) and suspended in sterile water just before inoculation. Killer colonies were identified by clear zones fringed with blue-stained dead cells, after incubation of the plates for 10 d at 17–20°C.

Growth and maximum biomass in selected media

Maltose fermentation tests were performed in Durham tubes (van der Walt and Yarrow 1984) at room temperature for 7 d and the production of gas and acid was monitored daily. The strains showing strong maltose fermentation were selected for further testing in different media at 28°C, in order to determine specific growth rates. Three media were used (Oda and Tonomura 1993): molasses medium containing 3% (w/v) total sugar as cane molasses, 0·193% (w/v) urea and 0·046% (w/v) KH_2PO_4; YPD medium (1% (w/v) Bacto-yeast extract, 2% (w/v) Bacto-peptone, 2% (w/v) glucose), and YPS medium (2% (w/v) Bacto-yeast extract, 4% (w/v) Bacto-peptone, 2% (w/v) sucrose, 0·2% (w/v) KH_2PO_4 and 0·1% (w/v) $MgSO_4 . 7H_2O$). Maximum biomass was determined for cells grown in YPS medium up to stationary phase and estimated in terms of dry weight. In order to confirm if all sucrose had been consumed, a sample was centrifuged at 12 000 rev min^{-1} for 2–3 min and the sucrose concentration was determined in the supernatant fluid following the reaction with β-fructosidase using a Boehringer Mannheim GmbH Sucrose/D-Glucose u.v. method test kit.

RESULTS AND DISCUSSION

Characteristics of the yeasts isolated

The yeast population present in the 33 dough samples studied comprised 73 isolates belonging to eight species (Table 1). No isolates of basidiomycetous affinity were found. The great majority were ascogenous yeasts. The predominant species, isolated from 80% of the samples, was *Saccharomyces cerevisiae*. Other yeasts occurred frequently, among which *Issatchenkia orientalis, Pichia membranaefaciens* and *Torulaspora delbrueckii* were the most abundant species, being present in about 40% of the doughs examined. Total yeast counts in the doughs were always between 10^5 and 10^6 cfu g^{-1}. These numbers were apparently independent of the dough composition and of the number and type of yeast species present.

Physiological characteristics considered relevant for yeasts to be used in the baking industry are summarized in Table

1. About 90% of the isolates were able to use lactate. Most of the isolates were also able to grow well at 42°C. The incidence of isolates able to grow at such high temperatures is probably the result of a natural selection related to the environmental conditions used in the preparation of the dough. In fact, traditional corn and rye bread is prepared with dough carried over from a previous making which is used as a starter. When the starter dough is mixed with the flour, the temperature inside the dough is high, never lower than 40°C.

Yeast associations

The distribution of the yeast populations among the 33 samples examined is shown in Table 2. Only 18% of the doughs consisted of a single yeast species. Associations of two species were found in 48% of the bread doughs, 30% presented three different species and the remainder consisted of a mixture of four yeast species. Associations of *S. cerevisiae* and *T. delbrueckii*, *Issatchenkia orientalis* and/or *P. membranaefaciens* were the most frequent. It is remarkable that all mixed populations included at least one typical fermentative species, either *S. cerevisiae* or *T. delbrueckii*, with the exception of the association between *P. anomala*, *P. membranaefaciens* and *I. orientalis* which was found in one of the doughs. The role of the latter species in the dough is probably correlated to the development of the organoleptic characteristics of the bread rather than with the fermentation of sugars. Apparently, this same dough is somehow similar to the San Francisco sour dough in which maltose-negative *S. exiguus* is predominantly found and the fermentation may be carried out by lactic acid bacteria (Sugihara *et al.* 1971). The dominant presence of *S. cerevisiae* might, in a few cases, be explained by the periodic re-introduction of compressed baker's yeast to accelerate the leavening of the products.

Most doughs analysed were made of a mixture of corn and rye or corn and wheat flours in different proportions. The highest pH value (4·25) estimated was found in the dough made of wheat flour with no other mixture. All the other pH values were lower and around 3·5. Furthermore, no relation

could apparently be established between the pH of the dough and the type of yeast association found. The results seem to indicate that the yeast population is rather specific of this particular substrate, since we found the same yeast associations in samples taken from doughs in geographically distant places and made of different types of flours.

Killer activity

The occurrence of killer activity in the yeasts isolated was also investigated. The presence of killer strains might be important to avoid the growth of contaminants and to establish a yeast population which contributes to the desirable organoleptic characteristics of the bread. As shown in Table 3, only about one quarter of the isolates behaved as killer strains under the conditions used. Strains of *P. anomala* displayed the strongest effects and the largest spectra, killing yeasts of the genera *Kluyveromyces, Pichia* and *Saccharomyces*. It is possible that the number of killer strains were underestimated due to the screening medium used. In fact, it was reported before that salt may be one of the important factors to influence toxin production in fermented foods (Suzuki *et al.* 1989; Marquina *et al.* 1992), and salt was not included in the medium.

Growth rate and biomass production

In order to evaluate the potential utilization for bread-making of the indigenous strains isolated, a preliminary screening was carried out based on their maltose fermentative capacity and biomass production. As it is well known, in lean doughs the principal fermentable sugar for yeast is maltose liberated from the starch of the flour by amylases, being generally accepted that a good baker's yeast should be able to rapidly ferment maltose (Burrows 1979; Evans 1990). Taking into account the results presented in Table 1, the strains of *S. cerevisiae* and *T. delbrueckii* showing strong maltose fermentation were further studied with respect to their growth rates and maximum biomass in different media in order to eliminate slow-growing and low-yielding strains. For comparison, three strains of *S. cerevisiae*, two isolated from commercial compressed yeast and one laboratory strain, as well as one strain of *T. delbrueckii*, were also tested. The results are shown in Table 4.

The most appropriate substrate for the growth experiments would be either cane or beet molasses which are used industrially to propagate baker's yeast. However, according to Oda and Tonomura (1993), there is considerable variation in the quality of molasses caused by an unpredictable fluctuation of their chemical composition, which can influence experimental data. Besides, we observed that growth in molasses medium under our laboratory conditions was difficult to assess correctly. To overcome these problems, we investigated the

effects of some media on the growth ability of the yeasts (results not shown). From our results we concluded that the most suitable medium to obtain the higher biomass yield for most of the tested yeasts, was YPS medium (pH 6·0). In terms of maltose fermentation and biomass values, two strains of *S. cerevisiae* and three of *T. delbrueckii* performed similarly or better than strains isolated from commercial compressed yeast, under the same conditions (Table 4).

Studies are progressing to test the leavening ability of the selected strains in dough and the influence of dough freezing and storage, during different periods, both on the viability of the yeast cells and on their fermentative capacity.

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TABLES

Table 1 Yeast species isolated from bread doughs and comparison of selected characteristics

Isolates	Number of strains	Glucose fermentation	Maltose fermentation	Galactose assimilation	Sucrose assimilation	Raffinose assimilation	Melibiose assimilation	Growth in lactic acid	Growth in 50% glucose	Growth at 42°C
Issatchenkia occidentalis Kurtzman *et al.*	1	D	–	–	–	–	–	+	–	–
I. orientalis Kudryavtsev	11	+	–	–	–	–	–	+	–	+
Kluyveromyces marxianus (Hansen) van der Walt	1	+	–	+	–	+	–	+	–	+
Pichia anomala (Hansen) Kurtzman	5	+	+	+	+	+	–	+	–	V
P. membranaefaciens (Hansen) Hansen	12	+	–	–	–	–	–	+	–	V
Saccharomyces cerevisiae Meyen *ex* Hansen	27	+	V	V	V	V	–	V	V	V
S. kluyveri Phaff *et al.*	3	+	D	+	+	+	–	+	V	+
Torulaspora delbrueckii (Lindner) Lindner	13	+	V	V	V	+	–	V	V	V

V, Variable; D, delayed, strong growth after 1 week or more.

Table 2 Number of yeast species present in 33 samples of doughs and their frequency of occurrence

Number of yeast species	Frequency of occurrence (number of doughs)	Frequency (%)
Single species	6	18
2 different species	16	48
3 different species	10	30
4 different species	1	4

Table 3 Killer activity in yeast isolates from home-made bread doughs against selected collection strains

Isolates	Sensitive strains							
	Number of strains	*Issatchenkia orientalis* IGC 3806	*Kluyveromyces marxianus* IGC 3886	*Pichia anomala* IGC 4380	*P. membranaefaciens* IGC 4619	*Saccharomyces exiguus* IGC 4612	*S. cerevisiae* IGC 4620	*Torulaspora delbrueckii* IGC 4978
Issatchenkia occidentalis	1	–	–	–	–	–	–	–
I. orientalis	11	–	–	–	+DB 51C DB 55D	–	–	+DB 33A
Kluyveromyces marxianus	1	+DB 44C	–	–	+DB 44C	–	–	–
Pichia anomala	5	–	+DB 50A	+DB 50A DB 50D DB 64B	–	–	+DB 50A	–
P. membranaefaciens	12	–	+DB 48A DB 50C DB 58B DB 60B	+DB 48A DB 50C DB 58B DB 60B DB 62B	–	+DB 33D	–	–
Saccharomyces cerevisiae	27	–	–	+DB 61C	–	–	–	–
S. kluyveri	3	–	–	–	–	–	–	–
Torulaspora delbrueckii	13	–	+DB 59C DB 61B	+DB 59C DB 61B	+DB 34D	–	–	–

+, Killer activity; –, no killer activity; positive results correspond to the strain designations indicated.

Table 4 Specific growth rate and maximum biomass of selected yeasts isolated in this study and of reference yeast strains in YPS medium (pH 6, 28°C)

Species	Strain	Growth rate (h^{-1})	Maximum biomass ($g\ l^{-1}$)
Saccharomyces cerevisiae	DB 52A	0·56	9·3
S. cerevisiae	DB 55A	0·67	10·6
S. cerevisiae	DB 55B	0·61	11·9
S. cerevisiae	DB 56A	0·64	10·0
S. cerevisiae	DB 56B	0·62	10·0
Torulaspora delbrueckii	DB 42B	0·51	12·3
T. delbrueckii	DB 48B	0·62	10·2
T. delbrueckii	DB 62A	0·53	13·2
S. cerevisiae	F 46A	0·60	10·6
S. cerevisiae	F 65A	0·70	11·6
S. cerevisiae	IGC 4072	0·63	10·4
T. delbrueckii	IGC 4978	0·53	10·3