Characterization and application of *Torulaspora delbrueckii* JK08 and *Pichia anomala* JK04 as baker's yeasts

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Summary

Baker's yeasts have long been known to be one of the oldest microbiological starter of food. The objective of study was to evaluate the potentials of *Torulaspora delbrueckii* JK08 and *Pichia anomala* JK04 as baker's yeasts. The cells are noticeably smaller and the growth was slower than that of *S. cerevisiae,* the conventional baker's yeast. These strains showed a lower leavening ability at an equal cells concentration. However, it tremendously increased when the cells concentration were raised, notably for *P. anomala* JK04. Interestingly, the strains showed remarkable tolerance to hyperosmotic and cation stresses. It was evident that the strains produced smaller bread loaf and consequently lower specific volume. This affected the textural characteristics of bread, as it had harder texture and greater chewiness. In contrast, the strains produced better springiness, brighter crust and crumb colour. The shelf-life of bread was comparable among the strains, except for *P. anomala* JK04, for which it was slightly shorter. According to the consumer evaluation, *T. delbrueckii* JK08 exhibited superior flavour, while *P. anomala* JK04 showed superior mouth feel. Despite of that, *T. delbrueckii* JK08 and *P. anomala* JK04 did not perform better in every aspect, employing its superiority in particular manner are greatly promising in bread making.

Keywords

baker's yeast; leavening ability; *Pichia anomala;* stress tolerance*; Torulaspora delbrueckii;* yeast morphology

Baker's yeasts are considered to belong to the oldest food microbial starters. Attention has been given more to baker's yeasts from 1927 to 2008. It was noted that 165 inventions out of over 337 baker's yeast strains have been patented [1]. Baker's yeasts are required in bread making to ferment the saccharides that are available in the flour or that have been added in formulation. Different source saccharides can be metabolized such as glucose, galactose, saccharose, maltose and one-third raffinose. The process produces carbon dioxide (CO_2), ethanol and other volatile compounds. CO_2, which is entrapped in the gluten matrix of the dough, causes dough leavening. At the same time, ethanol and other volatile compounds contribute to bread flavour and aroma after baking [2–4].

Saccharomyces cerevisiae has long been known as traditional baker's yeast. Strains of this species have been selected to produce bread of good quality as well as leavened and of good aroma. The yeasts can be marketed as compressed cakes, dry powder or instant yeasts [5–7]. Nowadays, new standard criteria for yeasts have been established due to the intention of producers to differentiate their product styles. It has led to the expansion of yeast selection to non-*S. cerevisiae* strains, with particular traits that are favourable for industrial applications [3]. Many efforts have been made to use non-*S. cerevisiae* or combination of *S. cerevisiae* with other yeasts or microorganisms. These included *Kluyveromyces marxianus* (NRRL-Y-2415 and NRRL-Y-1109), which were assessed for its leavening ability in lean dough and rich dough, and comparing them with *S. cerevisiae* [8], the co-cultures of *Lactobacillus fermentum*, *S. cerevisiae* and *Candida krusei* used as starter cultures of the Ghanaian maize dough [9], kefir grains used as baker's yeasts in lean dough [10], kokja or nuruk (Korean traditional starter) applied to

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sponge and dough [11], *Torulospora delbrueckii* used in sweet and sweet frozen dough [12, 13], and *Pichia anomala* SKMT used as starter in white pan bread [14].

Recently, *T. delbrueckii* has attracted a great attention in wine and bread industry since it has particular traits such as producing a specific flavour [15], forming less alcoholic fermentation by-products [16], high fermentation purity and producing low levels of undesirable aroma compounds such as hydrogen sulphide and volatile phenols [17], having resistance to environmental stress [13] and higher leavening ability [12]. However, yet it has not been produced and implemented consistently in bread industry due to limited knowledge of its genetic and physiological background compared to the well known baker's yeast *S. cerevisiae* [3].

P. anomala (Hansen) was characterized and named by KURTZMAN in 1984. It used to be named *S. anomalus, Hansenula anomala, Monilia javanica, Mycoderma cereuisiae, Candida javanica* and several other names [18]. It is also recognized as naturally occurring biocontrol agent similar to *Metschnikowia pulcherrima, Debaryomyces hansenii* and *P. guilliermondii* [19]. It was shown to produce a killer toxin, which prevented mould spoilage and enhanced preservation of moist grain due to malfunction of storage systems [20], as well as reduced Enterobacteriaceae in inoculated barley [21]. *P. anomala* is a non-*Saccharomyces* yeast in alcoholic beverages, being applied to enhance the sensory quality by producing particular volatile compounds [22–24]. In the latest publication, it was reported that its application as baker's yeast in white-pan bread enhanced the bread aroma and prolonged the shelf-life, inhibiting moulds [14]. However, the application of *P. anomala* in brewing and baking industries is still in the beginning phase. In addition, there is limited knowledge on applicability of *P. anomala* as baker's yeast.

The objective of the present study was to characterize and evaluate the potential of *T. delbrueckii* JK08 and *P. anomala* JK04 as baker's yeasts. A comprehensive evaluation of this potential included characterization of its morphological properties, growth curves, stress tolerance, leavening ability, and its influence on bread quality and shelf-life.

MATERIALS AND METHODS

Strains, chemicals and ingredients

The yeasts *T. delbrueckii* JK08 and *P. anomala* JK04 were obtained from the Institute of Fermentation Biotechnology, Kyungpook National University (Daegu, South Korea). The yeast *S. cerevisiae* was isolated from compressed instant baker's yeast (saf-instant S.I. Lesaffre, Marcq, France). Yeast extract-Peptone-Dextrose (YPD) broth and agar (technical) were purchased from Difco (Le Pont de Claix, France) and Oxoid (Hampshire, United Kingdom), respectively. Sodium chloride (NaCl) and potassium chloride (KCl) were from Duksan (Ansan, South Korea). Sorbitol was from Daejung Chemical (Shiheung, South Korea) and lithium chloride (LiCl) was from Yakuri Chemical (Kyoto, Japan). Commercial wheat flour (Beksul, CheilJedang, Seoul, South Korea) specified for bread making was purchased online. Sugar and salt were purchased from supermarket in Sangju, South Korea.

Cultivation and enumeration of cells

The yeasts were grown by inoculating the colonies aseptically into 5% YPD broth in a clean chamber. Then, these were cultivated in rotary shaker (JSSI-300C, JS Research, Gongju, South Korea) at 30 °C and 18.84 rad·s^{-1} for 48 h. The cells number was enumerated using hemocytometer (Neubauer chamber; Celeromics, Cambridge, United Kingdom). After growing in 5% YPD broth, the yeast mixture was centrifuged (Hanil Supra 22K, Hanil, Incheon, South Korea) at 4000 ×*g* for 10 min at 4 °C. The supernatant was discarded and the sediment was re-suspended in distilled water and vortexed thoroughly. This yeast mixture was stored at 2–4 °C for further experiments. To enumerate the cells, 1 ml of the yeast mixture was taken and 10^{-1}–10^{-4} dilutions were prepared. The Neubauer chamber was observed at 100× magnification by using binocular microscope (CX31RTSF, Olympus, Tokyo, Japan). The cells number from 4 different areas in the chamber was enumerated sequentially. Then, the cells number was calculated using the following formula:

$$c = \frac{N}{n \cdot d} \times 10\,000 \quad (1)$$

where c is concentration (expressed as number of cells per millilitre), N is number of cells, n is number of squares and d is dilution. Number 10000 represents a constant converter to millilitre.

Morphology of yeast cells

Yeast mixture (100 μl) obtained from the previous section was placed onto a slide and covered. This was air-bubble free to minimize noise during image processing. A binocular electric microscope (Eclipse TS100, Nikon, Tokyo,

Japan) equipped with image grabber software (TS View ver. 7.3.1.7, Xintu Photonic, Fujian, China) was used to grab picture of yeast colonies at 400× magnification. For cell area measurement, the method introduced by PONGJARUVAT et al. [25] and COELHO et al. [26] were used with modifications. Yeast image was analysed by using ImageJ software (ver. 1.47, National Institutes of Health, Bethesda, Maryland, USA). For image processing, the image was adjusted for brightness and contrast to clearly distinguish between the object and background. The binarization was then carried out to produce binary images. Prior to area measurement, the circularity was set to 0.25–1 and the area size was varied to one another considering the smallest and the largest area in binary images. Finally, the area size distribution chart was constructed using Microsoft Excel 2007 (Microsoft, Redmond, Washington, USA).

Correlation between optical density and cells numbers

Correlation between cells number and its optical density (*OD*) was studied by enumerating cells population at a series of dilutions. *OD* was measured with spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan) at 600 nm (OD_{600}). Six dilutions were used to generate the curve. The cells were enumerated as described above. Then, the cell numbers and OD_{600} of the suspension were plotted onto a scatter plot and a linear regression was generated by Microsoft Excel.

Growth curve

Growth curve was constructed by measuring the time-course evolution of OD_{600} of liquid cultures [13]. Firstly, 500 ml of 5% YPD broth was inoculated with yeasts. Then, the yeast mixture was incubated in an incubator shaker at 30°C and 18.84 rad·s^{-1} for 48 h. The *OD* of the cultures was measured in every 2 h for 12 h using a spectrophotometer (Shimadzu). Afterwards, this was measured every 4 h up to 48 h. The obtained data were plotted onto a line chart showing the growth curve of yeasts.

Stress tolerance

Stress tolerance was tested by the method described by HERNANDEZ-LOPEZ et al. [13]. The yeasts were cultivated as described in the previous section. Stress tolerance was tested on 5% YPD agar containing 2 mol·l^{-1} sorbitol, 2 mol·l^{-1} NaCl, 2 mol·l^{-1} KCl, and 0.8 mol·l^{-1} LiCl. The agar plates containing those compounds were stored overnight prior to inoculation. Serial dilutions of the yeast mixture (1–10^{-4}) were made. The yeast mixture (3 μl) was taken from every dilution and spotted consecutively onto agar plates. The plates were then incubated in an incubation chamber (Lab Companion IL-11-2C; Jeio Tech, Seoul, South Korea) at 30 °C for 48 h.

Leavening ability

Leavening ability of yeasts was measured by the method described by CABALLERO et al. [8] and PLESSAS et al. [10] with a slight modification. The yeasts were added for assessing the leavening ability were at approx. 1×10^8 cells per ml and 4×10^8 cells per millilitre. A blend containing 20 g flour, 20 ml water and yeasts was mixed thoroughly in a 100 ml graduated cylinder, followed by incubating at 30 °C for 210 min in an incubation chamber (Jeio Tech). Observation was carried out in every 30 min. Maximum leavening rate (in millilitres per hour) was calculated from the highest volume reached in 210 min, divided by the time in hours at which the highest volume was first recorded.

Bread making

Standard baking method optimized for bread maker (National SD-BT102, Panasonic, Osaka, Japan) was applied for producing white pan bread. The standard bread formula containing 280 g wheat flour, 16.8 g sugar, 5.6 g salt and 200 ml water, at 28–30 °C, was applied. Yeast cultures were prepared by dissolving 5×10^8 cells per millilitre of water for *S. cerevisiae*, while for *T. delbrueckii* JK08 and *P. anomala* JK04, approx. 10^9 cells per millilitre of water were added. Dry ingredients were weighed precisely and poured into a 5 l polyethylene (HDPE) bag. The blend was mixed manually by hand shaking for 30 times followed by transferring into bread maker's bucket. Subsequently, this was mixed by rotating the bucket for 10 times. The 4 h standard bread making specified for white pan bread was employed. Baking was performed in triplicate. After baking, the bread loaves were tempered at ambient temperature (28–30 °C), and then subjected to further analyses.

Shelf-life of bread

The shelf-life of bread was determined according to PLESSAS et al. [10] with modifications. Freshly baked bread was cut with a slicer machine, which was sterilized using 70% alcohol. Three slices of bread from each treatment were handled and put aseptically into HDPE plastic bag and tied tightly. Then, these were stored at 30 °C and evaluated every day. The shelf-life was evaluated by observing the incidence of moulds on the bread slices. The day prior to the incidence of moulds on

the bread slice was considered as bread shelf-life.

Physical and sensory evaluation of bread

Physical evaluations were carried out including moisture content, specific volume, bread yield, crust and crumb colour and textural profiles. The moisture content was determined by the oven-drying method [27]. Specific volume was determined by seed displacement method AACC 10-05 [28]. Bread yield was calculated according to the method of MOVAHED et al. [29]. The crust and crumb colour were measured using chromameter CR-400 (Konica-Minolta, Tokyo, Japan), *L* (lightness), *a* (redness), and *b* (yellowness) values being recorded. Six points upon surface of bread crust and crumb were measured. Whiteness index (*WI*) was calculated according to HSU [30] and LIN et al. [31]. Bread texture profile analysis (TPA) was carried out in triplicate and 3 different points of bread crumb surface were measured by the method of ULZIIJARGAL [32] and SKARA [33] using TA-XA2 texture analyzer (Stable Micro Systems, Hamilton, Massachusetts, USA) with a slight modification. Bread samples were cut to slices of about 25 mm thickness, then subjected to measurement of hardness, cohesiveness, springiness and chewiness. The settings and conditions for TPA test were: 38 mm acrylic cylindrical probe, 2 mm·s^{-1} of pretest speed, 2 mm·s^{-1} of test speed, 10 mm·s^{-1} of post-test speed, 10 mm of distance, 50 g of force and 3 s of time.

Sensory evaluation was carried out according to ULZIIJARGAL [32] with slight modifications. Bread samples were prepared from fresh baked loaf (6–8 h after baking). The bread was cut to pieces of approx. 50 × 20 × 25 mm and served on a small paper plate. Sensory evaluations were conducted by fifteen undergraduate students at the Department of Food and Food-Service Industry, Kyungpook National University, South Korea. The sensory attributes assessed included appearance, colour, flavour, mouthfeel and overall acceptability. Seven-step scale of sensory attributes was used for assessing the bread, whereby 1 was extremely dislike, 4 was neither like nor dislike and 7 was extremely like.

Statistical analysis

To examine the significance among treatments, the data were analysed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) at the level of significance $p < 0.05$. The analysis was carried out using SPSS for Windows ver. 19 (IBM, New York, New York, USA).

RESULTS AND DISCUSSION

Morphology of yeast cells

The analysed cell were cell shape, area size and area size distribution. The images of *T. delbrueckii* JK08, *P. anomala* JK04 and the reference yeast *S. cerevisiae* cells are presented in Fig. 1, while, the cell area size distribution profiles are shown in Fig. 2. It can be seen that the cells shape of *S. cerevisiae* was subglobose and ovoidal. The cells were occurring in pairs with the daughter cell or forming small clusters. For *T. delbrueckii* JK08, the cells shape was spheroidal, the cells occurred singly and randomly forming short chains. *P. anomala* JK04 cells were cylindrical or ellipsoidal and occurred in pairs with daughter cells or singly. It has been noted that the cells shape of *S. cerevisiae* is globose, ovoidal or elongate and the cells are usually isolated or in small clusters after 3 days at 25 °C in 5% malt extract. *T. delbrueckii* cells are well known to be spheroidal to ellipsoidal, occuring singly or oc-

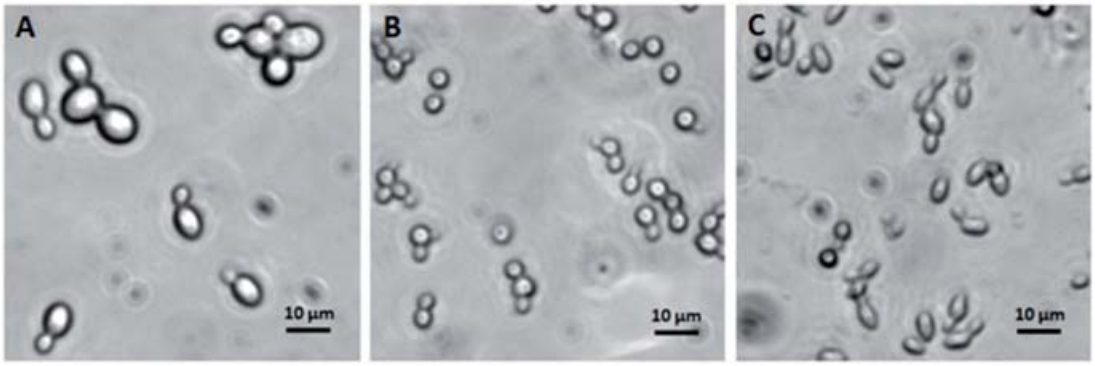

Fig. 1. Yeast cells imaged by microscopy.

A – *S. cerevisiae*, B – *T. delbrueckii* JK08, C – *P. anomala* JK04.

casionally in pairs after 3 days at 25 °C on 5% malt extract agar. *P. anomala* cells were reported to be spheroidal to elongate, occurring singly, in pairs or forming small clusters [18]. Thus, observations of the present study regarding cell morphology were in agreement with the previous study.

In this present study, we also compared the cells size by projecting them in binary images and analysed using ImageJ software. The obtained data were subjected to measurement of cell area and cell area distribution (Fig. 2). In descending order, the means of cells area for *S. cerevisiae*, *P. anomala* JK04 and *T. delbrueckii* JK08 were 42.56 μm^2 > 20.73 μm^2 > 17.91 μm^2, respectively. If assumed as perfectly spherical, the mean cell diameter would be 7.36 μm > 2.57 μm > 2.39 μm, respectively. This illustrates that *S. cerevisiae* cells were definitely bigger than the other two, while *T. delbrueckii* JK08 cells were slightly bigger than *P. anomala* JK04. From the data shown in Fig. 2, *S. cerevisiae*, *T. delbrueckii* JK08 and *P. anomala* JK04 cells area were in the range of 10–85 μm^2, 5–30 μm^2, and 5–45 μm^2, respectively. For *S. cerevisiae*, population was dominated by cells having a small area (10–55 μm^2). On the contrary, population of *T. delbrueckii* JK08 was dominated by those having cell area of nearly mean size (15–25 μm^2). *P. anomala* JK04 population was dominated by cells with a small area and nearly mean size (5–30 μm^2). Cell size was measured as a ratio of cell width and length axis, surface area, and volume. Cells of *S. cerevisiae* are classified into three different groups as follows: a large type (4.5–10.5 μm) × (7.0–21.0 μm), an intermediate type (3.5–8.0 μm) × (5.0–11.0 μm) and a small type (2.5–7.0 μm) × (4.5–11 μm) [34]. Apparently, *S. cerevisiae* used in the present study belonged to the intermediate type. The size of *P. anomala* JK04 cells was also in agreement with the preceding study, which reported the cells size to be (1.9–4.1 μm) × (2.1–6.1) μm. On the contrary, the cell size of *T. delbrueckii* JK08 was slightly smaller than that reported by the previous study which was (2.1–5.6 μm) × (3.0–6.6) μm [18]. It was reported that the cell size of particular yeast strains is affected by many factors, such as ploidy [13, 35, 36], growth conditions (temperature, media, stress), growth cycle, and the age of the individual cell [26, 34].

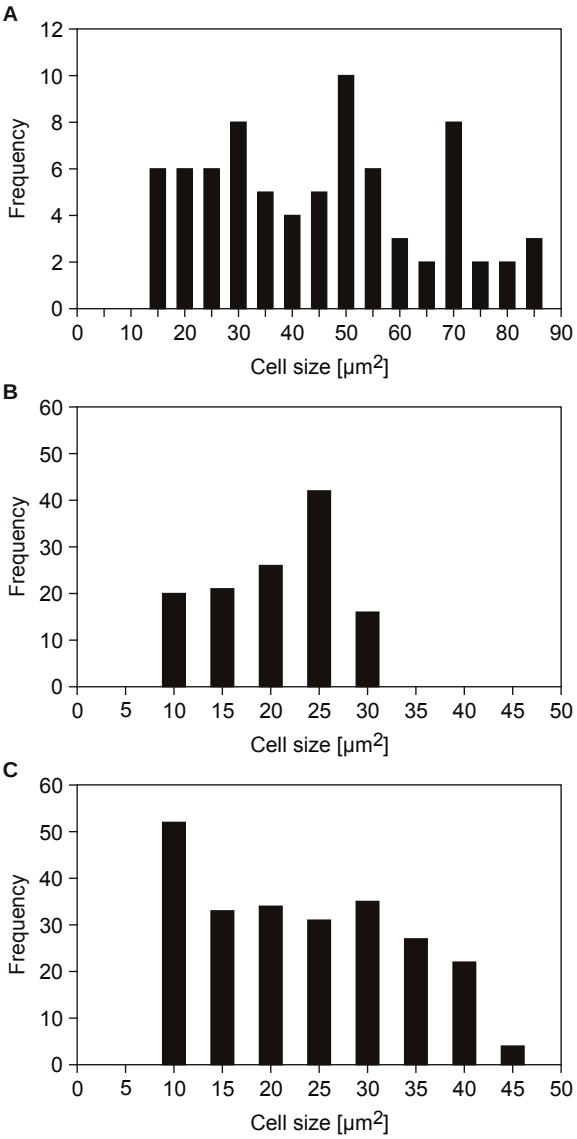

Fig. 2. Histogram showing the distribution of cell area sizes.

A – *S. cerevisiae*, B – *T. delbrueckii* JK08, C – *P. anomala* JK04.
The total cell numbers of *S. cerevisiae, T. delbrueckii* JK08 and *P. anomala* JK04 that could be rendered and analysed from sample images were 76, 125 and 238 cells, respectively.

Correlation between optical density and cells numbers

For $OD < 1.0$, the cell numbers and OD were highly correlated. In this range, $OD_{600} = 1.0$ is equivalent to approx. 3×10^7 cells per millilitre [37]. Fig. 3 shows correlation between OD_{600} and cell numbers of different yeasts. We demonstrated that at $OD_{600} = 1.0$ yeast cell numbers were different for different yeast strains. For *S. cerevisiae*, *T. delbrueckii* JK08 and *P. anomala* JK04, the cells numbers were approx. $1.39 \times 10^7 < 2.41 \times 10^7 < 3.25 \times 10^7$ cells per 1 ml, recpectively. Determination of cell numbers on the basis OD_{600} is convenient, as it is fast, but it cannot discriminate between the dead and live cells. The determination depends on cell size and geometry and so

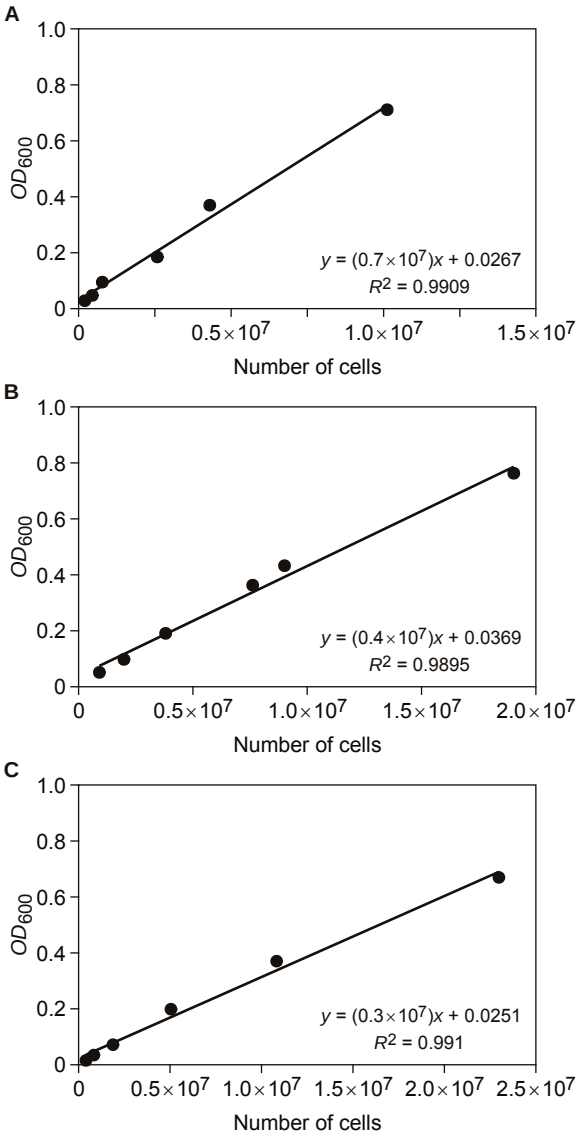

Fig. 3. Correlation between OD_{600} and cell numbers.

A – *S. cerevisiae*, B – *T. delbrueckii* JK08, C – *P. anomala* JK04.

construction of a calibration curve is necessary to determine cells population by using OD_{600} [36]. As aforementioned, at $OD_{600} = 1$, the cells number of *S. cerevisiae*, *T. delbrueckii* JK08 and *P. anomala* JK04 are different. We have confirmed that the cell size and the geometry of these yeasts are different as shown in Fig. 1. Thus, these affected the light to be scattered and resulted to different *OD* value at an equal number of cells.

Growth curve

It can be seen in Fig. 4 that *S. cerevisiae* grew markedly faster than the other two yeasts. The lag phase of *S. cerevisiae* was shorter than those of *T. delbrueckii* JK08 and *P. anomala* JK04. The lag phase of *T. delbrueckii* JK08 being the longest. The logarithmic phases of *S. cerevisiae*, *P. anomala* JK04 and *T. delbrueckii* JK08 were initiated after 2 h, 10 h and 16 h, respectively. Despite that *T. delbrueckii* JK08 initiated the logarithmic phase later, it grew to a greater density than the other two yeasts (Fig. 4).

For baker's yeasts, growth rate is critical as the yeast is needed for leavening. After mixing all ingredients, the yeast action is needed immediately to break down starch and saccharides, and then to ferment it. Afterwards, the carbon dioxide enters the dough cells causing the dough to leaven. Consequently, abundant amounts of yeast cells are needed in order to produce a properly leavened dough. Generally, yeast cells can grow rapidly in a rich medium containing bactopeptone and yeast extract. The growth rate of yeasts depends on temperature. The cells multiply well at room temperature, but more rapidly at 30 °C. At this temperature, wild-type yeasts can divide approx. every 90 min in a rich medium [36, 37]. Regarding the growth rate, *S. cerevisiae* was superior to the other two yeasts. *S. cerevisiae* is a traditional baker's yeast and has been selected and optimized for baking application, and particular traits were developed by the yeast manufacturer [3]. In contrast, *T. delbrueckii* JK08 and *P. anomala* JK04 are newly introduced baker's yeasts, which have to be studied and improved extensively.

Stress tolerance

The ability to adapt to deleterious environments is a challenge for baker's yeasts. We compared the tolerance of the yeasts by growing in YDP medium containing stressor substances related to industrial processes. The stress against high osmotic conditions were tested by growing the yeasts in the culture medium containing 2 $mol \cdot l^{-1}$ sorbitol and 2 $mol \cdot l^{-1}$ KCl. The yeasts were also subjected to stress conditions against cations Na^+ and Li^+ [13]. Generally, *S. cerevisiae* were less tolerant to all stress conditions compared to the other yeasts (Fig. 5). As can be seen, the cells could not maintain its growth in agar medium with high concentrations of sorbitol, NaCl, KCl and LiCl. The yeasts grew poorly in the presence of sorbitol, even poorer in the presence of KCl or LiCl, and are completely inhibited in the presence of NaCl. *T. delbrueckii* JK08 and *P. anomala* JK04 grew comparably in all stress conditions. Apparently, *T. delbrueckii* JK08 was more tolerant to stress caused by the cations, meanwhile, *P. anomala* JK04 was more tolerant to high osmotic stresses (Fig. 5). The ability to adapt to

deleterious effects during bread making is imperative for baker's yeasts. Despite that, *S. cerevisiae* being used as baker's yeast for ages, has some parameters far from optimal [3]. It was reported that the effect of Na^+ and K^+ on the growth of *S. cerevisiae* varied in respect with other factors such as temperature and pH. It was shown that *S. cerevisiae* was less adaptive to Na^+ and K^+ at supra-optimal temperature and at different pH compared to *Debaryomyces hansenii* [38]. In addition, the cells of *T. delbrueckii* showed osmotolerance and ion toxicity tolerance superior to *S. cerevisiae* in bread dough containing 20% saccharose and 2% salt [12]. On the contrary, PETROVSKA et al. [39] reported that *S. cerevisiae* exhibited higher osmotolerance than *P. anomala* upon addition of 5–20% glucose and 4% NaCl. It is obvious that hyperosmolarity provokes reduction of cell volume, loss of turgor and growth inhibition [40]. Monovalent cations such as Na^+ and K^+ are very important in yeast metabolism. Potassium is needed in the regulation of cell volume and intracellular pH as well as enzyme activation. Consequently, this cation accumulated in cells at fairly high concentrations. Nonetheless, accumulation of sodium or its analogue lithium and potassium at high levels is toxic to cells [41, 42].

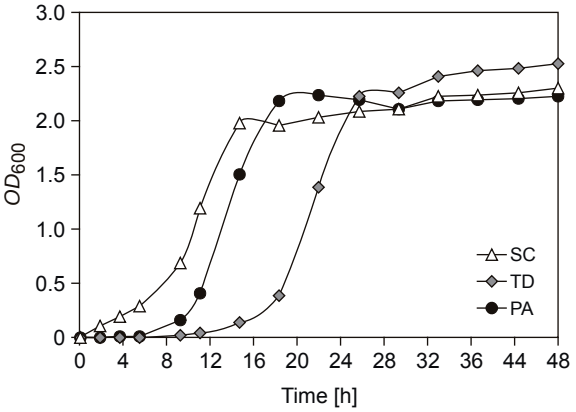

Fig. 4. Growth curves of yeasts.

SC – *S. cerevisiae*, TD – *T. delbrueckii* JK08, PA – *P. anomala* JK04.

Leavening ability

Leavening ability is the most important property of baker's yeasts [3]. We investigated the leavening ability of *S. cerevisiae*, *T. delbrueckii* JK08 and *P. anomala* JK04 in a dough containing wheat flour and water. We found that the leavening ability of *S. cerevisiae* was noticeably greater than that of *T. delbrueckii* JK08 and *P. anomala* JK04 (Fig. 6). The leavening rates of *S. cerevisae*, *T. delbrueckii* JK08 and *P. anomala* JK04 were 34.50 $ml \cdot h^{-1}$ > 5.43 $ml \cdot h^{-1}$ > 0.52 $ml \cdot h^{-1}$, respectively. These were achieved by using yeasts at approx. 1×10^8 cells per millilitre. When the concentration of the yeasts was increased to 4×10^8 cells per millilitre, the leavening rates were 42.00 $ml \cdot h^{-1}$ > 8.67 $ml \cdot h^{-1}$ > 2.29 $ml \cdot h^{-1}$. By increasing the yeast concentration, the leavening ability was greatly improved. As the yeast concentration was increased about 4 times, the leavening ability of *S. cerevisiae*, *T. delbrueckii* JK08 and *P. anomala* JK04 increased by approx. 20%, 60% and 340%, respectively. Thus, boosting the leavening ability is possible by increasing the yeast concentration. The worse performance

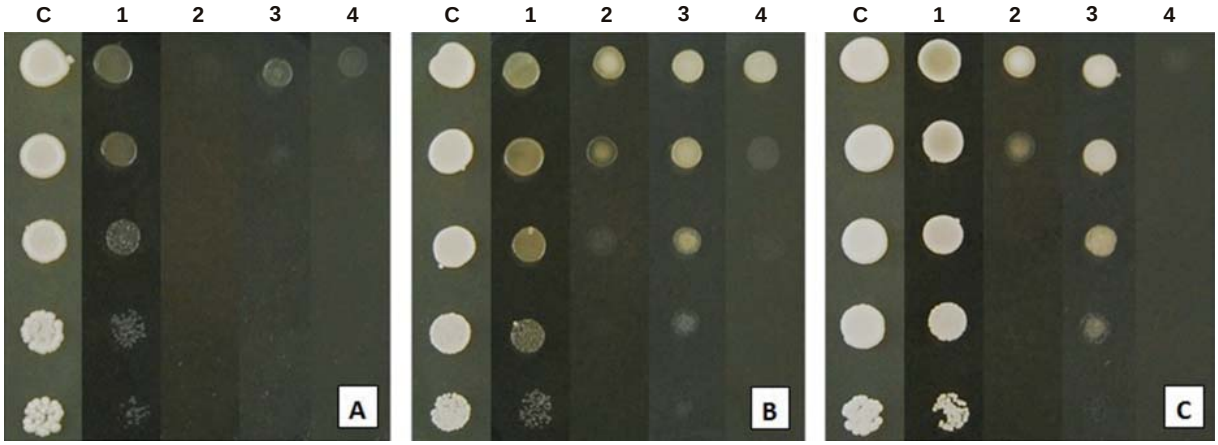

Fig. 5. Tolerance of the yeasts to stresses.

A – *S. cerevisiae*, B – *T. delbrueckii* JK08, C – *P. anomala* JK04.
C – control, 1 – 2 $mol \cdot l^{-1}$ sorbitol, 2 – 2 $mol \cdot l^{-1}$ NaCl, 3 – 2 $mol \cdot l^{-1}$ KCl, 4 – 0.8 $mol \cdot l^{-1}$ LiCl.

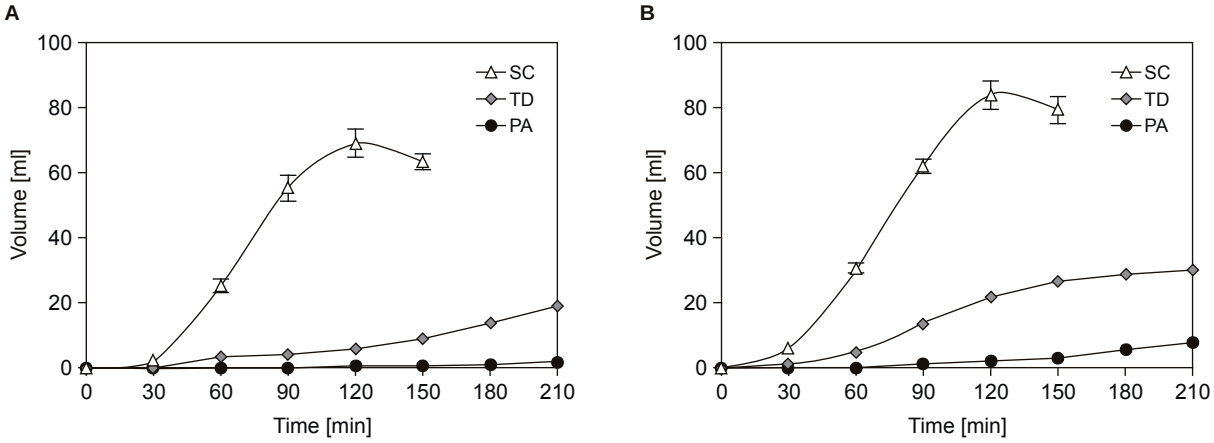

Fig. 6. Ability of the yeasts to leavening the dough.

A – inoculated with 1×10^8 cells per 1 ml of water added; B – inoculated with 4×10^8 cells per 1 ml of water added. SC – *S. cerevisiae*, TD – *T. delbrueckii* JK08, PA – *P. anomala* JK04.

of *T. delbrueckii* JK08 and *P. anomala* JK04 might have been due to a slower growth (Fig. 4). In addition, the strains also produced CO_2 at a lower rate compared to *S. cerevisiae* (data not shown). This was in agreement with the study by Almeida and Pais [43], who reported a higher leavening ability in commercial *S. cerevisiae* strains (IGC 5325 and IGC 5326) than those in *T. delbrueckii* (IGC 5321 and IGC 5323). However, this was in contrast with a previous study, which reported a significantly higher CO_2 production rate in *T. delbrueckii* than in *S. cerevisiae* in a medium with saccharose at 30 °C [44]. On the other hand, *T. delbrueckii* (IGC 5321 and IGC 5323) showed higher leavening ability than *S. cerevisiae*, especially after exposure to hyperosmotic stress in the dough containing 20% saccharose and 2% salt [12].

Physical properties and shelf-life of bread

Breads leavened with studied yeasts were baked and their physical properties and shelf-life were evaluated (Tab. 1). The differences in moisture contents of breads leavened with studied yeasts were insignificant. The bread loaves leavened with different yeasts are shown in Fig. 7. It can be seen that the bread loaf volume among the yeasts was markedly different, the specific volumes being significantly different ($p < 0.05$). The bread leavened with *S. cerevisiae* had the highest specific volume (4.74 ± 0.19 $cm^3 \cdot g^{-1}$), followed by *T. delbrueckii* JK08 (3.79 ± 0.05 $cm^3 \cdot g^{-1}$) and *P. anomala* JK04 (3.55 ± 0.01 $cm^3 \cdot g^{-1}$). This was in accordance with the leavening ability of *S. cerevisiae*, which was superiority to the other two yeasts (Fig. 6). Further, it might have been also due to a longer lag phase of *T. delbrueckii* JK08 and

Fig. 7. Bread loaves leavened with different yeasts.

A – *S. cerevisiae*, B – *T. delbrueckii* JK08, C – *P. anomala* JK04.

Tab. 1. Physical properties and shelf-life of breads leavened with *S. cerevisiae*, *T. delbrueckii* JK08 and *P. anomala* JK04.

	Yeast		
	S. cerevisiae	*T. delbrueckii* JK08	*P. anomala* JK04
Moisture [%]	59.4 ± 3.2 a	56.6 ± 3.4 a	53.3 ± 3.8 a
Specific volume [$cm^3 \cdot g^{-1}$]	4.74 ± 0.19 a	3.79 ± 0.05 b	3.55 ± 0.01 c
Bread yield [%]	144.4 ± 0.1 b	148.1 ± 1.3 a	149.6 ± 0.2 a
Crust colour			
L	38.09 ± 1.85 b	42.74 ± 0.59 a	41.38 ± 3.13 ab
a	7.33 ± 2.71 a	9.14 ± 0.56 a	8.92 ± 0.40 a
b	16.86 ± 0.33 b	20.15 ± 0.30 a	19.34 ± 1.49 a
Whiteness Index	35.38 ± 2.09 a	38.61 ± 0.56 a	37.59 ± 2.51 a
Crumb colour			
L	56.03 ± 1.50 b	55.92 ± 1.04 b	59.14 ± 1.78 a
a	–1.66 ± 0.01 c	–1.22 ± 0.02 b	–1.05 ± 0.03 a
b	10.15 ± 0.37 c	11.78 ± 0.34 b	12.71 ± 0.17 a
Whiteness Index	54.84 ± 1.38 ab	54.35 ± 0.98 b	57.20 ± 1.65 a
Texture profile			
Hardness [g]	829.24 ± 59.77 b	1 935.99 ± 118.56 a	2 264.44 ± 412.57 a
Springiness	1.00 ± 0.00 b	1.60 ± 0.01 a	1.50 ± 0.11 a
Cohesiveness	1.00 ± 0.00 a	0.46 ± 0.05 b	0.53 ± 0.03 b
Chewiness [g]	829.24 ± 59.77 b	1 429.11 ± 234.25 a	1 787.29 ± 281.62 a
Shelf-life [d]	3	3	2

Values are means ± standard deviation, $n = 3$.
Means with same superscript letter in a row are not significantly different ($p < 0.05$), determined by Duncan's multiple range test.

P. anomala JK04 (Fig. 4). As reported by HERNANDEZ-LOPEZ et al. [12], a longer lag phase would reduce maximal loaf volume. In term of bread yield, *S. cerevisiae* exhibited the lowest value, which was significantly different from the other two yeasts. The higher weight loss of bread during baking due to moisture loss leads to its better quality [29].

Regarding the crust colour, the *L* value for *T. delbrueckii* JK08 was the highest (42.74) and significantly different ($p < 0.05$) from that of *S. cerevisiae* (38.09), but insignificantly different from *P. anomala* JK04 (53.34). The a values did not differ significantly among the yeasts. The *b* value of *T. delbrueckii* JK08 did not differ significantly from that of *P. anomala* JK04. However, the two were significantly higher ($p < 0.05$) than that of *S. cerevisiae*. The *WI* value of the crust did not differ significantly among the breads produced with the studied yeasts. For the crumb colour, the *L, a, b* values of the bread prepared with *P. anomala* JK04 were the highest and significantly different ($p < 0.05$) from the others. The *WI* value of this was significantly different ($p < 0.05$) from the bread prepared with *T. delbrueckii* JK08, but not significantly different from the bread prepared with *S. cerevisiae*. The colour formation in the crust and crumb is greatly affected by non-enzymatic reactions, so-called browning. These reactions involve saccharides and nitrogen-containing substances, which create a dark coloured pigments melanoidins [7]. The noticeably darker colour may also be due to a greater maltase and invertase activities, which produce more reactive saccharides. It has been reported that the commercial baker's yeast (*S. cerevisiae*) exhibited greater maltase and invertase activity than *T. delbrueckii* IGC 5321 [12].

The hardness, springiness and chewiness of breads leavened with *T. delbrueckii* JK08 and *P. anomala* JK04 were significantly greater ($p < 0.05$) than that of *S. cerevisiae*, however, the cohesiveness was significantly lower ($p < 0.05$) (Tab. 1). The higher hardness and chewiness values of breads prepared with *T. delbrueckii* JK08 and *P. anomala* JK04 may due to their lower leavening and gassing abilities, which resulted in a less developed bread. The cohesiveness and springi-

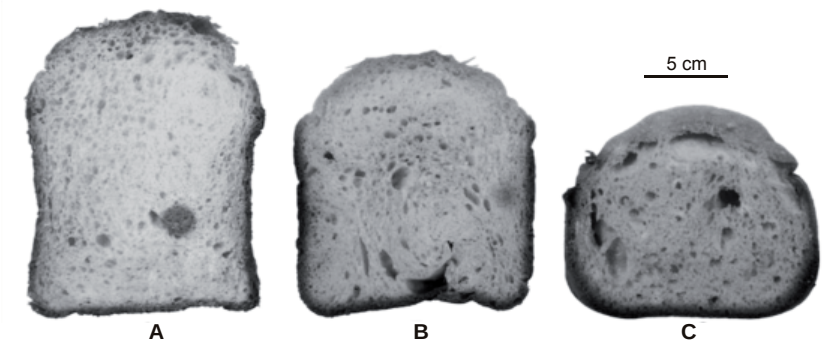

Fig. 8. The incidence of moulds on bread slices during storage.

A – *S. cerevisiae*, B – *T. delbrueckii* JK08, C – *P. anomala* JK04.

ness are generally considered as positive properties, but not hardness and chewiness [45]. These descriptors are interrelated with the cells structure of bread such as the wall thickness, cell size and uniformity. The combination of these parameters largely determine textural properties of the crumb. Finer, thin-walled and uniformly-sized cells produce a softer texture [46]. Hardness, the loss of softness, is a negative attribute of most bakery products. Generally, the greater the loaf volume, the lower will be its hardness value [45]. It is evident that *S. cerevisiae* had the greatest specific volume, which corresponded to its lowest hardness value. The crumb cohesiveness is controlled by moisture content and strength of networks surrounding the cells pore. In short, cohesiveness is the ability to form a ball or to stick together immediately in the mouth. On the other hand, chewiness represents the energy required to break up food for swallowing. In addition, springiness represents elasticity and related to the strength of cell wall network of crumbs, hence the fresh bread is expected to be springy [32, 45].

The shelf-life was determined on the basis of mould growth on bread slices (Fig. 8). We found that the shelf-life of bread prepared with *P. anomala* JK04 was slightly shorter than of the other two. It was only 2 days for *P. anomala* JK04, and 3 days for *S. cerevisiae* or *T. delbrueckii* JK08. This result was different from the previous study, which reported an extended shelf-life of a bread leavened with *P. anomala* SKMT [14]. Several studies reported on the biocontrol activity of *P. anomala* against yeasts, moulds and bacteria due to its killer toxin [20, 21]. Specifically, Izgu et al. [47] reported that *P. anomala* produced a killer toxin, which was a glycosylated protein. The toxin showed high stability at pH 3–5.5 and at temperatures up to 37 °C. The results obtained in this study can be explained by the lost of the biological activity of the toxin due to high temperature during baking.

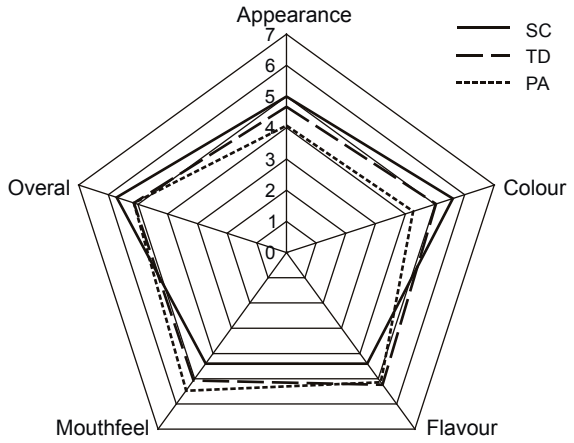

Fig. 9. Radar plot of sensory properties of bread leavened with different yeasts.

SC – *S. cerevisiae*, TD – *T. delbrueckii* JK08, PA – *P. anomala* JK04.

Sensory properties of bread

The sensory properties of bread were evaluated and the results were formulated in a radar plot (Fig. 9). Generally, the sensory properties of breads prepared with different yeasts were comparable. All attributes were satisfactory in the range from 4.07 to 5.71. The overall sensory score of bread leavened with *S. cerevisiae* was 5.71, and 5.14 for *P. anomala* JK04 and *T. delbrueckii* JK08. *S. cerevisiae* was superior on overall acceptability, which might have been based on a better appearance (5.00) and colour (5.60). Bread prepared with *T. delbrueckii* JK08 was superior on flavour (5.23), and bread prepared with *P. anomala* JK04 was superior on mouthfeel (5.47). It seems that the

consumers preferred a moderately dark crumb, as confirmed by moderate lightness (L) value for *S. cerevisiae* (Tab. 1). Better appearance might have been due to a properly leavened bread, which contributed to a better crumb structure. Based on these results, we suggest that *T. delbrueckii* JK08 and *P. anomala* JK04 are suitable to be used as baker's yeasts, which could produce better flavour and specialty taste.

CONCLUSIONS

T. delbrueckii JK08 and *P. anomala* JK04 have a comparable cell size, but smaller than *S. cerevisiae*. The strains showed slower growth and lower leavening ability than *S. cerevisiae*. However, these yeasts showed remarkable tolerance to hyperosmotic and cation stresses. Employing these yeasts as a leavening agent significantly affected the physical and sensory properties of bread. The strains did not perform better as baker's yeasts in all criteria compared to the commercial yeasts but, in some aspects, they showed superiority. Thus, *T. delbrueckii* JK08 and *P. anomala* JK04 are largely promising as candidates of emerging baker's yeasts with specialty characteristics.

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