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Effect of the temperature and osmotic stress on the growth and cell viability of *Candida maltosa*

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Abstract

The present research attempted to observe the temperature and osmotic stress effects on yeast species in cheese. The *Candida maltosa* yeast was isolated from locally made raw cheese samples, and it was identified by its biochemical properties, followed by using the BIOLOG™ microbial identification system to confirm species level identification. The study investigate about *Candida maltosa*'s ability to grow and develop a budding pattern under high temperatures up to 44°C and osmotic stress for 72 h. Osmotic stress tolerance was studied at 32.5°C in different concentrations of dextrose (0.04 g/l, 0.12 g/l, 0.2 g/l, 0.28 g/l, and 0.36 g/l) and sucrose (0.02 g/l, 0.06 g/l, and 0.36 g/l), respectively. Cell growth of the *C. maltosa* was measured by optical density at 600nm (OD_{600}) and the enumeration of colony forming units (CFUs) on the agar plates up to 300 m. The obtained result indicated the optimal growth pattern at 32.5°C and complete growth retardation at 44°C with the high concentrations of dextrose (0.36 g/l) and sucrose (0.38 g/l). Particularly, *C. maltosa* budding could only survive 240 m at 37°C to 40°C and was suppressed at 44°C. The experimental findings demonstrated the stress response in yeast cells in the phenotypic level with the existing acquaintance on the osmotic stress response actions in *C. maltosa.*

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Introduction

Yeasts comprise a large and diverse group of microorganisms having considerable biological significance and presently increased interest to the scientist of the world (Martins *et al*. 2014). Their abundance and various biological functions make them promising candidates for food and other sectors, including cheese and bread preparation, alcohol fermentation, and biological control (Psani and Kotzekidou, 2006; Afrin *et al.* 2021). The majority of yeast species belong to Ascomycotina; very few are basidiomycetes. There are almost 500 known species of yeast, but *Saccharomyces cerevisiae* is the only well-studied yeast in terms of biotechnological applications and how it responds to different environmental stresses (Tofalo *et al*. 2007; Vernocchi *et al*. 2011; Schuller *et al*. 2012). *C. maltosa* is a unicellular, oval-shaped, diploid yeast (Komagata *et al*. 1964). *C. maltosa* is well-known among *Candida* species for its ability to grow on a wide range of substrates, such as carbohydrates, fatty acids, and n-alkanes. *C. maltosa*'s physiology, biochemistry, and molecular genetics are well studied. As a result, it has grown huge importance in terms of commercial interests. This species can change its shape based on its environment because it is dimorphic and hydrophobic (Calderone and Fonzi, 2001). Long-term environmental changes can cause stress, which can influence genetic changes in living beings (Deak, 2006; Drumonde-Neves *et al*. 2016; Afrin *et al*. 2021). The earlier reports indicated that diverse changing environments influence the growth and function of microorganisms (Kusumi *et al*. 2013; Ikeda *et al*. 2014; Yamaguchi *et al*. 2014). Responses to stress are especially important for microbes that live in places where conditions like temperature, osmotic stress, and the availability of nutrients are always

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changing (Estruch, 2000; Afrin *et al*. 2020). Microorganisms can respond to environmental stresses in many different ways, such as by making spores and cysts, making molecules or growth factors to relieve stress, changing the cellular membrane, releasing damaged enzymes that have been fixed, and so on (Bhuiyan *et al*. 2016; Storz and Hengge, 2011). Earlier studies suggested that yeast cells could survive and adapt to unfavorable conditions (Claro *et al*. 2007). Turkel *et al*. (2000) illustrated examples of how osmotic stress, heat shock, and oxidative stress affect *S. cerevisiae*'s growth ability to regulate its gene expression. The cell division of yeast cells is highly influenced by heat stress and osmotic shock (Rowley *et al*. 1993; Morano *et al*. 2012). Heat stress is a condition that can create stress in yeast cells, alter their physiology, or even cause death. Oxidative stress is a type of environmental stress that refers to an interaction between two concentrated solutes that are separated from one another by a permeable membrane (Pratt *et al*. 2003; Verghese *et al*. 2012). According to other studies (Pratt *et al*., 2003; Morano *et al*. 2012; Van Wuytswinkel *et al*. 2000; Yamamoto *et al*. 2007), a very high osmotic pressure, such as high sugar concentrations, may alter yeast metabolism or reduce viability. Earlier research (Beney *et al*. 2001; Bond, 2006; Gibson *et al*. 2007) discovered that when yeast cells are, subjected to heat and osmotic stress, their cells break down and their capacity to divide slows down. Although the model experimental yeast species *S. cerevisiae* was well recognized for its osmotic and thermal stress responses, little was known about other yeast species. Osmotic pressure, pH, oxidative stress, heat, and other factors might have impacts on the intracellular process of yeast, which is used in commercial processes like manufacturing cheese. To study how different yeast species react to heat shock and osmotic stress, the major objectives of this investigation were to isolate and identify the various yeast species present in cheese samples. We believe that the current work is the earliest investigation of the *C. maltosa* yeast species from cheese in Bangladesh. These findings will offer understanding of various stressors placed on *C. maltosa*. These new findings will give us novel insights into how *C. maltosa* responds to different stresses, like temperature and osmotic pressure.

Materials and methods

Culture media, chemicals, and samples

All microbiological media used were from Hi-Media, India. Biochemical reagents and all other chemicals are from Sigma, USA; the lactophenol cotton blue reagent is from Thermo Fisher Scientific, India. Ten (n = 10) different cheese samples were collected in sterilized giplock bag from the local market of Dhaka, Bangladesh, in March 2019. After collection the samples were brought immediately to the laboratory, labeled properly and maintained at 4°C both before and after usage.

Isolation of yeast strain

Each sample was subjected to the pour plate procedure to identify potential yeast strains. The plates were incubated for three to five days at 30°C using Sabouraud dextrose agar (SDA) medium. Yeast colonies were seen following the incubation time. The selected isolates were purified using the repeated streaking technique and stored at 4°C until further use.

Phenotypic characterization of the strain

According to the previous reported method, yeast strains were observed with an optical microscope (Olympus, Japan) (Anderson and Soll, 1987). With the help of an inoculating loop, a thin layer of yeast culture is teased on a clean slide, and a drop of lactophenol cotton blue is also added and left for a few minutes. The slide was examined under the microscope using low-power and subsequently with high-power objectives.

Biochemical methods for yeast identification

Following morphological characterization, the following biochemical tests such as: gelatin liquefaction, urea hydrolysis, acid formation from sugars, exploitation of nitrogen, fermentation, carbon supply, and H_2S production (Van der AaKühle *et al*. 2003; Jespersen, 2003) were conducted for identification purpose.

Identification of yeast by BIOLOG™ System

The Yeast isolate was identified at the species level using the BIOLOG™ identification system (BIOLOG™, USA). All chemicals, reagents, and growth media were purchased from the same company. According to Kostas *et al*. (2019), this method uses various carbon sources attached to a 96-well microtiter plate together with a positive control and a negative control to identify the strain of yeast. The yeast strain was grown on Biolog universal yeast agar (BUY) medium for 24 h at 32.5°C and. Then, yeast strain was added to the inoculating fluid, which had already been heated, to get the desired turbidity of 44–51% T. For the assimilation and oxidation studies, a range of dehydrated carbon sources was employed, including glucose, sucrose, galactose, lactose, maltose, cellobiose, L-sorbose, raffinose, melibiose, trehalose, inulin, soluble amides, melezitose, D-xylose, D-ribose, L- and D-arabinose, methanol, D-glucosame, N-acetyl-D-glucosame, glycerol, ethanol, erythitol, ribitol, sodium

citrate, DL-lactic acid, galactitol (dulcitol), D-mannitol, D-sorbitol, salicin, methyl-D-glucoside, L-rhamnose, D-gluconic acid, lysine, L-arabinitol, sodium succinate, butane 2.3 diol, inositol, ketoglutaric acid, hexa-decanexylitol, propane (1.2 diol), ethylame, creatine, cadaverine, potassium nitrate, and glucosame. The other experiments were conducted by fermentation of sugars such glucose, sucrose, galactose, maltose, trehalose, melezitose, lactose, cellobiose, and inulin to observe sugar utilization pattern.

The yeast cell suspension was prepared by adding the yeast cell to the inoculating fluid and placed into reservoir. To inoculate in all 96 wells, 100 µl of the cell suspension were drawn up by repeating pipettor and put on microplate, thus covered with its lid and kept incubating for 24 h at 32.5°C. After incubation, the microplate was put into the Micro Station Reader for analysis to get the yeast strain ID, and the result was obtained by comparison with the database using the software application Micro Log 4.20.05 (BIOLOG™, USA) (Kostas *et al*. 2015; Kostas *et al*. 2019). The combination of the 96 assay reactions and the complicated explanatory software reveals a high level of precision comparable to molecular methods.

Heat response analysis

Cells respond to a variety of environmental stresses by adapting to a specific strain that is heat sensitive. The optimum temperature range for yeast is 32°C to 35°C. Cells of *C. maltosa* were put though both mild and deadly heat stress, and their ability to live was checked. The cultivation temperature was increased from 30°C to 44°C. *C. maltosa* grows best at 32.5°C, but it can grow in temperatures as high as 37°C. A loop-full colony from the freshly cultured yeast species was used to form a pre-culture in 5 mL SDB, which was then incubated at 30°C, 32.5°C, 37°C, 40°C, and 44°C, respectively in stagnant conditions for 72 h. The growth of colony forming units (CFUs) and the optical density at OD_{600nm} were monitored at specific intervals. The critical growth temperature was determined by monitoring growth at 40°C and 44°C, respectively. To check the morphological view, 5 µl of each culture was removed at 60 m intervals (Munna *et al*. 2015). In order to achieve up to a 10-fold concentration for the spot dilution test, 1 mL of yeast culture suspension was serially diluted in 9 ml of dextrose and sucrose broth separately (Nur *et al*. 2014).

Osmotic pressure analysis

Sucrose and dextrose sugar were used to examine the osmotic pressure tolerance of several yeast species (Munna *et al*. 2015). The CFUs on the agar plates were counted, and the optical density was estimated to OD_{600nm}. An aliquot of 5 µl was taken from each dilution and put on SDA plates. The plates were kept at 32.5°C for 24 h. Dextrose and sucrose were used in a variety of concentrations to study the impact of osmotic pressure on cell growth, including sucrose (0.02 g/l, 0.06 g/l, 0.1 g/l, 0.14 g/l, and 0.18 g/l) and dextrose (0.04 g/l, 0.12 g/l, 0.02 g/l, 0.28 g/l, and 0.36 g/l), respectively.

Statistical analysis

During experiment, tests were conducted with 03 (three) replicates for having statistical analysis and the data were reported as the mean (+/-) standard deviation (SD).

Results and discussion

Culture characteristics and microscopic study

The typical yeast cell seems to be small circular colony with a smooth surface, an entire boundary, a glossy white color, a convex elevation and opaque opacity (Beney L *et al.* 2001). In this study, only one isolate was found to exhibit similar morphological phenomenon, as a result, this isolate initially selected for further study. The morphological characteristic of the chosen strain was assessed by an optical microscope. The isolated colony was found to have smooth surfaces with circular margins, a creamy white color, and was oval and/or spherical in shape (Fig. 1A). Lactophenol cotton blue staining was observed under high power objective, it showed clear budding (Fig. 1B). In previous study, Calderone and Fonzi (2001) found the identical morphological view about *Candida* species.

Biochemical characteristics of the isolate

Important biochemical tests were carried out for the provisional identification of the yeast strain.The isolated strain was capable of fermenting D-glucose, Maltose and Sucrose but not Lactose. The strain also had the ability to ferment Galactose. The strain also demonstrated success in melibiose fermentation. The selected yeast species passed the tests for using L-lysine and potassium nitrate while not using niacin or thiamine. Additionally, the strain performs poorly in the tests for the synthesis of starch-like compounds, urea breakdown, and cycloheximide (Table I). In previous studies, Anderson JM and Soll DR (1987) observed that *Candida* sp. strain showed the comparable biochemical characteristics. In the same way, Calderone RA and Fonzi WA (2001) also found the similar test result like present study.

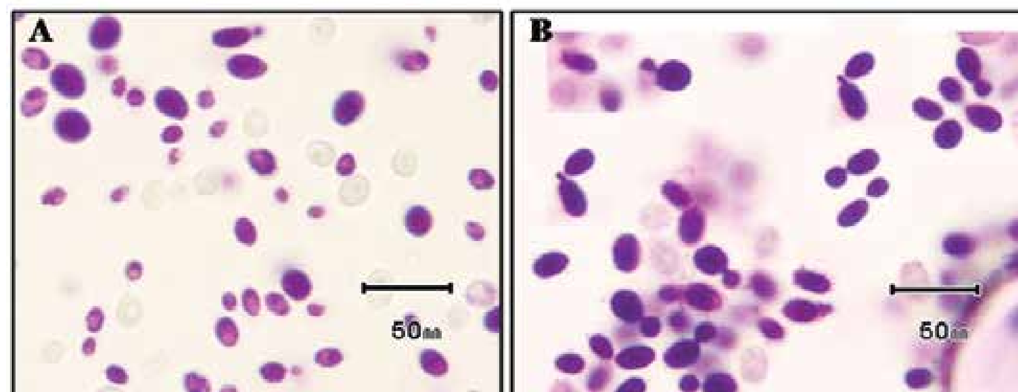

Fig. 1. Analysis of the morphology of *C. maltosa* cells (A). Spherical or oval shapes when using low-power objectives (B). Light microscope observation of *C. maltosa's* active budding

Table I. Biochemical characteristics of the *C. maltosa* strain

Tests	Results	Tests	Results
Fructose	+	Melibiose	+
Sucrose	+	Potassium nitrate	+
Maltose	+	L-lysine	+
Lactose	-	Non-niacin	+
Starch	-	Non-thiamine	+
Galactose	+	Urea hydrolysis	-

Identification of yeast species using the BIOLOG™ microbial identification system

A similarity index was used to choose the yeast colony based on how the yeast reacted on microplates. For yeast identification, a reading with a similarity index value of 0.5 or higher at 48 to 72 h is also necessary, as is an appropriate similarity index value of 0.75 or above at 24 h (Praphailong *et al*., 1997). The yeast species that were consistently identified as *C. maltosa* in the database's species library (Micro LogTM 4.20.05, Biolog, USA) had a 75% possibility and a 0.5 similarity index value. Three replicates of the yeast strain were looked at for additional confirmation. Table II and the supplemental Fig. 1 provide a summary of the findings.

Expression of *C. maltosa* in response to environmental stresses

Heat response analysis

The temperature tolerance of the *C. maltosa* strain is presented in Fig. 2. The effect of heat stress was studied at various temperatures for varying lengths of time (24 h, 48 h, and 72 h). By measuring the OD_{600nm} and counting CFUs from 0 to 300 m, the standard growth temperature was calculated. When compared to higher temperatures, the growth rate of strain *C. maltosa* was substantially faster at 32.5°C, However, the growth was more moderately stable at 30°C. On the other hand, a very long lag phase (120 m) was noticed following the yeast strain's growth at 37°C to 40°C. This may have begun because the yeasts were exposed to higher temperatures for a longer period of time than they would have under ideal growth conditions. It has already been demonstrated that yeast cells can grow at temperatures as high as 40°C without displaying any evidence of cell lysis, which could be caused by the hard cell wall and cell membrane (Munna *et al*. 2015). But under a microscope, yeast began to form after 120 m at 30°C and 32.5°C. The C. maltosa yeast strain lost its activity at 44°C. The absorbance and CFU cell count were not particularly high at 44°C. *C. maltosa*'s critical growth temperature is thus 44°C.

Osmotic stress analysis of C. maltosa

According to the earlier reports, the yeast cells possibly showed an instantaneous growth arrest, while exposed to an increase in external osmolality. The osmotic component of the cell was demonstrated in survival tests that were conducted by varying the amount of sugar added to SD broth (Morai-

Table II. Yeast strain (Y-1) identification with BIOLOG^{TM} identification system

Yeast Strain	Probability	Similarity	Distance	Strain ID
Y - 1	0.795	0.552	1.068	*Candida maltosa*

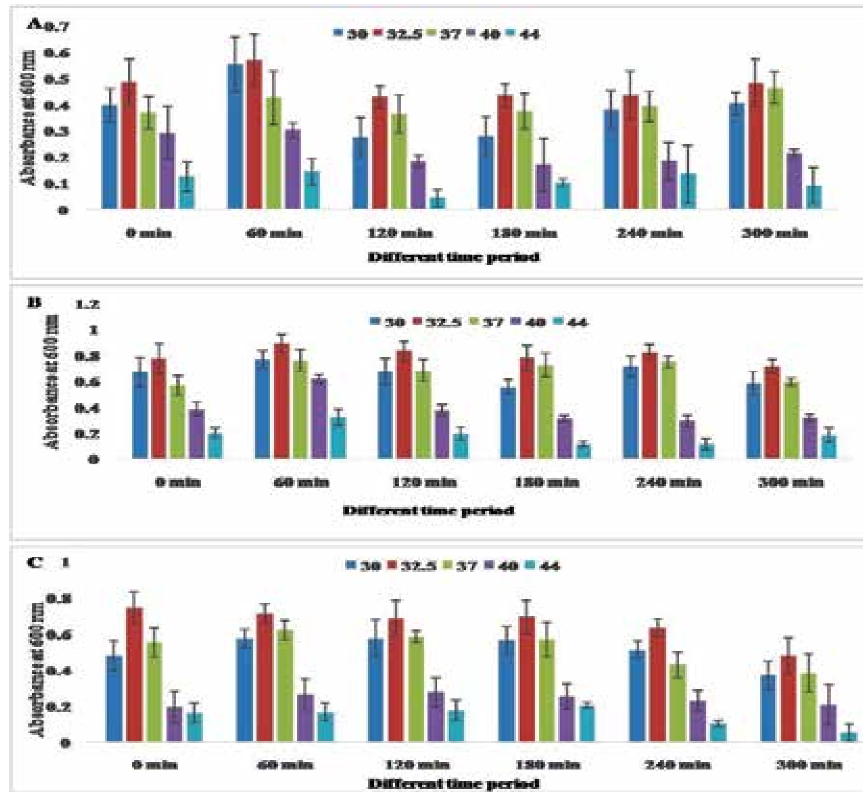

Fig. 2. Shows a morphological analysis of *C. maltosa* cells at various temperatures incubation using a OD_{600nm} absorbance measurement (A) after 24 h, (B) after 48 h, and (C) after 72 h, respectively

tis and Curran, 2004; Haruta and Kanno, 2015). It appears that the dynamics of the outside osmotic pressure are crucial for a cell to remain alive based on how osmoregulation functions. Different concentrations of sucrose (0.02 g/l, 0.06 g/l, 0.1 g/l, 0.14 g/l, and 0.18 g/l) and dextrose (0.04 g/l, 0.12 g/l, 0.02 g/l, 0.28 g/l, and 0.36 g/l) were employed to assess *C. maltosa*'s tolerance to sugar. At 32.5°C, there was a relatively long lag phase (120–240 m) in both the dextrose and sucrose concentrations. By estimating the optical density at OD_{600nm} and counting the CFUs on the agar plates for up to 300 m, cell growth was seen. So, a constant force kept pushing this dextrose and sucrose water into the cell along its concentration gradient. The reading was taken at the end of the 24 h incubation period, which indicated 0 m. In the dextrose sugar experiment, at 1X (240 m), C. maltosa tolerated the most and gave the highest peak of absorbance, and at 7X (0 m), this strain showed the lowest tolerance. At the same time, *C. maltosa* was blooming at its normal temperature in a 9X concentration. In the same way, *S. cerevisiae*'s tolerance for dextrose went from 7X to 9X over time (Munna *et al*. 2015). *C. maltosa* had the highest absorbance in sucrose at 7 x (240 m) (Fig. 3A). Therefore, in sucrose, it can thive more than in dextrose. In both sugar experiments that resulted in the observation of morphological changes, a long lag phase was observed. Similar to *S. cerevisiae*, in 5X dextrose concentrations were dormant, and yeast *C. maltosa* thickened in higher concentrations (Fig. 3B). Because of the osmotic pressure, at different concentrations of sugar, the yeast changes its shape. Under osmotic pressure, the "starch body" inside the yeast changed and made up a larger part of the yeast as a whole. In 9X (60 m) dextrose concentration, yeast structure was successively extended in size, and budding occurred until 180 m; after that, it went dormant again. Yeast cells displayed a

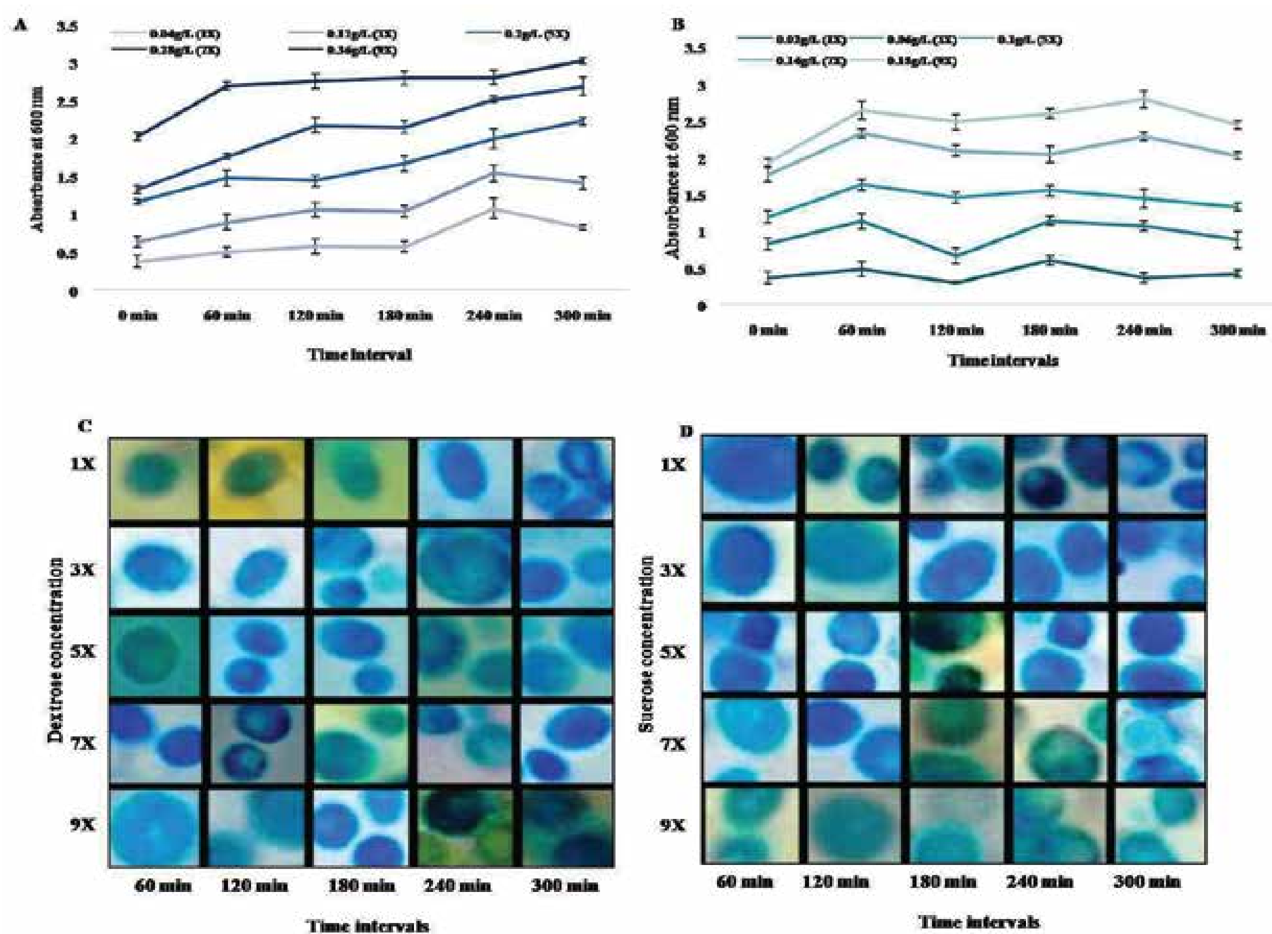

Fig. 3. (A). Growth of *C. maltosa* at various dextrose concentrations, (B). Growth at various sucrose concentrations, (C). Morphological studies at various dextrose concentrations, and (D). Morphological studies at various sucrose concentrations

prolonged structure and continued to bud in sucrose 1X (60 m) until 7X (Fig. 3D) osmotic pressure was reached.

Pratt *et al*. (2003) found that these situations showed that yeast cells could maintain osmotic pressure or tolerate an osmotic imbalance in cells for a longer time. It was discovered to grow in certain incubation phases when compared to *S. cerevisiae* at 32.5°C with different sucrose concentrations (Morano *et al*. 2012). According to this physiological mechanism, it is not genetic, because the cells would show the same growth-related osmotic hypersensitivity in the SDA medium if they were put into new, living colonies that were growing under stressful conditions. The impact of osmotic stress or pressure was dependent on the strain. This information provides some valuable strategies for C. maltosa against osmotic stress. The results of this study show that yeast can handle or tolerate sucrose at a 7X concentration very well. On the contrary, dextrose showed less tolerance at 1X concentration compared to sucrose. This means that at the best temperature, *C. maltosa* can handle a higher concentration of sucrose than dextrose. Moreover, this yeast strain showed a linear stationary phase in both dextrose and sucrose sugar for 300 minutesof incubation.

Conclusion

The critical temperature for the growth of the yeast strain *C. maltosa* was discovered to be 40°C. At temperatures exceeding 40°C, it started to go dormant or slow down its growth. This yeast strain enters its death phase around 44°C, where it is unable to live. Additionally, an important aspect of how yeast functions can be significantly impacted by experiments involving heat stress, sugar tolerance, or osmotic pressure. By observing the changes to the shape of yeast cell, osmotic pressure analysis indicating that the *C. maltosa* strain can

handle sucrose better than dextrose. The important discovery was that the yeast strain could grow at 44°C but that adding high sugar concentrations enabled growth to be stopped.

Conflict of interest

There is no conflict of interest stated by the authors.

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Data availability statement

On request, the corresponding author will deliver the experimental information that underlies the study's findings.

Ethics statement

There are no human studies or animal experiments in this article.

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