EXPLORATION OF POTENTIAL YEAST (*Candida maltosa*) FROM MOZZARELLA CHEESE AND ITS PROSPECT FOR PROBIOTIC ACTIVITY

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelor of science in biotechnology

Department of Mathematics and Natural Sciences

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January,2020

Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing a degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all the main sources of help.

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Approval

"Exploration of Potential Yeast (*Candida maltosa*) from Mozzarella Cheese and its Prospect for Probiotic Activity" submitted by
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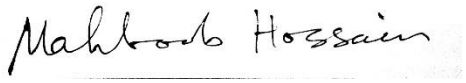

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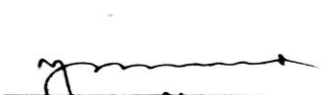

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Abstract

To be a good probiotic any bacteria or yeast need to fulfill some requirements. There are some tests need to be conducted both in-vitro and in-vivo. Probiotics are living organisms which administered in adequate amounts to giving a beneficial health effect on its host. Besides the probiotic study, some other experimental observations were conducted. The optimal temperature of *Candida maltosa* was 32.5℃, pH was measured where the optimal pH was 7 with the highest absorbance of 1.194 and at 3% saline concentration *C.maltosa* showed its optimal salinity tolerance. Furthermore, to check some activity such as heat stress analysis where the extreme temperature at (40℃ and 44℃) eliminates the growth of *Candida maltosa*. Osmotic stress identifies the tolerance potentiality of *Candida maltosa* at different sugar concentrations, where in dextrose 1x (0.04g/L) desired organism showed its highest tolerance and in sucrose 7x (0.14g/L) it gave the highest tolerance. Morphological patterns were seen in both heat stress and osmotic stress activity. The artificial gastric juice and bile salt tolerance of *C.maltosa* was investigated at pH of 2, 2.5, 3, 3.5, 4, and 4.5 and salt tolerance at the concentrations of 0.1%,0.5% and 1% where *C.maltosa* showed its potentiality in both tests.

Key word: *Candida maltosa*; temperature; pH; salinity; heat stress; osmotic pressure; probiotic activity; bile salt; gastric juice.

Acknowledgement

I would like to express my deepest appreciation to the Almighty for his continuous blessings and providing me with the strength, good health and patience to be able to carry out and complete this research study.

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List of Acronyms

ml: Milliliter

µl: Microliter

g/L: Gram per Liter

e.g.: For example

et al: And others

pH: Negative logarithm of hydrogen ion concertation

CFU: Colony Forming Unit

Spp.: Species

%: Percentage

℃: Degree Celsius

nm : Nanometer

Chapter 1

Introduction

1.1.1 Microorganism: Yeast (*Candida maltosa*)

Candida maltosa, isolated from mozzarella cheese. *Candida maltosa* is a unicellular, oval-shaped diploid fungus. The organism is dimorphic and, under different environmental conditions, changes from one morphological to the next (Calderone *et al*., 2001).

Because of the hydrophobicity of all cell surfaces, it forms biofilms on the surface of technical equipment (Wikipedia, 2019). The species can be isolated from the spoiled yogurt fruit surface. Besides, *C.maltosa* has a quantified effect on its production of lactic acid (Wikipedia, 2019). The organism also has glucose tolerance to acids. Besides, *Candida maltosa* has also risen in glucose with 1.6% of lactic acid. *C.maltosa* indicates that it has probiotic activity through the acid, and bile salt tolerance process. This species can thrive on a wide range of substrates, including n-alkanes, fatty acids and carbon dioxide. *C.mlatosa* has been intensively studied regarding its physiology of growth and composition of biomass (Wolf, 1996).

1.2.2 Heat stress and osmotic pressure analysis:

With a previous screening of bacterial cellular responses to heat stress and osmotic stress, current research has further explored this effect on yeast cells (Munna *et al.*, 2015). Heat stress includes several situations in which the body becomes overheated under stress. Increased temperature causes changes in the system of gene expression, composed of an immediate modification of the transcription of 'early stress response genes', often referred to as the environmental stress response (ESR), which is a common response to many stresses (Caspeta *et al.*, 2016). Followed by improvements in the expression of genes unique to the heat stress response (Caspeta *et al*., 2016). Following the magnitude and domination of heat stress, changes in the genomic expression can be unstable and finally adjusted to ensure that the cells can confront the new temperature *(*Caspeta *et al*., 2016).

Microorganisms like yeast, *Candida maltosa* are developing systems to counteract the effect of osmotic stress such as salt stress (NaCl) (El-Moghaz *et al*., 2010). The current study tried to observe the influence of high temperatures (40-46℃) on growth and budding patterns of *Candida maltosa.* (Munna *et al.*, 2015). Also observed was the effect of elevated concentration of sugar as another stress stimulant (Munna *et al*., 2015)

Osmotic pressure refers to a force that arises between two different concentrated solutes that separated by a semipermeable membrane (Pratt *et al.*, 2003). When yeast is exposed to sugar it is subjected to osmotic pressure. Very high osmotic pressure such as high sugar concentration may deform yeast metabolism or diminish yeast viability (Pratt *et al*., 2003). The intensity of the osmotic pressure depends on the concentration of solutes around the cell (Pratt *et al.*, 2003). In many ways, yeast responds to the effects of the concentrations of solute on the growth medium. In response to osmotic pressures, yeast cell adjusts their cell volume, decrease the volume in response to hypertonic stress and the presence of hypotonic stresses (Pratt *et al.*, 2003). For osmotic pressure analysis, dextrose and sucrose should be in a favorable concentration to maintain an appropriate volume and ratio of cells (Munna *et al*., 2015).

Every living cell must observe the adaptation ability of free water (Hohmann *et al.*, 2002). The most underlying principles in yeast are osmotic adaption (Hohmann *et al.*, 2002). Yeast cell wall has the capacity of sensing and signaling the osmotic stress (Hohmann *et al*., 2002).

1.2.3 The probiotic potentiality of *Candida maltosa*

The probiotic activity has been observed on this strain of *Candida maltosa.* Much effort has been given to obtain elevated *C.maltosa* strain with probiotic and technological properties in the food and dairy industry (Nagyzbekkyzy *et al.*, 2016).

In many studies, the result s of probiotics are contradictory; most of them prove that they are beneficial while other studies suggest that they have no beneficial effects (Jin *et al.*, 2003). The reason why the results differed might be due to the strain of the microorganism used. Therefore, the emphasis was placed on some factors for the preparation and selection of *Candida maltosa* strain as probiotic (Havenaar *et al.*, 1992). For the selection of probiotics, certain factors, such as bile tolerance and gastric juice tolerance, must be considered which will generate beneficial results.

Probiotic, a phrase derived from Latin, means ‘for life’. a long term earlier than the awareness of probiotic microorganisms, fermented products, which include beer, bread, wine, kefir, kumis, and cheese were very often used for the dietary and therapeutic purposes (Ozen *et al*., 2014) Probiotics are non-pathogenic micro-organisms that exert a fine when consumes in sufficient quantities, affect the health of their host (Nagyzbekkyzy *et al.*, 2016). According to the World Health Organization, “Live organisms which when administered in adequate amounts confer a health

benefit on the host". Scientific evidence for the health benefit would need to be recorded in order to be labeled a probiotic (Mack *et al.*, 2005). Although most of the common microorganisms used as probiotics are of the genus *Lactobacillus* and *Bifidobacterium,* another genus of bacteria, including *Enterococcus, Streptococcus* and *Escherichia* are used. The fungus *Saccharomyces boulardii* is also considered a probiotic (Mack *et al.*, 2005)

A variety of micro-organisms, especially lactic acid bacteria (LAB) have been evaluated for his or her probiotic ability and are implemented in various varieties of meals merchandise or in healing preparations (Nagyzbekkyzy *et al.*, 2016). Some of the beneficial effects of probiotic bacteria or yeast includes: (i) improving the health of the intestinal tract;(ii) improving the immune system, synthesizing and improving the bioavailability of hypersensitive responses in inclined individuals; and (iv) decreasing the danger of certain cancers (Parvez *et al*., 2006). Unlike the other organism like yeast, *Candida maltosa* has also been shown its probiotic activity during the bile salt and gastric juice assay. This organism had been isolated from mozzarella cheese. The purpose of this study was to identify the activity of potential probiotic from *Candida maltosa* and provide some data for the development and utilization of probiotic in the near future.

To check the probiotic activity, two in-vitro analysis had been done.

Artificial Gastric juice tolerance assay:

Gastric juice had been produced in-vitro. This analysis is one of the major experiments of, observation of probiotic activity. Promising yeasts' activities, as well as their ability to survive through the human gastrointestinal tract, have acknowledged their potential use as probiotics (Hattingh *et al.*, 2001). Besides, few studies have focused specifically on selecting or testing new strains of yeast (Agarwal *et al.*, 2000). Probiotic yeast must survive transit through the stomach before reaching the intestinal tract and be subjected to gastric acid constituents, which is the primary mechanism of defense against most ingested micro-organism (Marteau *et al.*, 1993)

Bile salt tolerance assay:

Bile salt tolerance is an important assay in *C.maltosa* for probiotic activity observation. *C.maltosa* was able to survive, to grow and strive for its action in the small intestine. When a probiotic reach into the intestine, its survival depends on their resistance to bile. Although intrinsic bile tolerance appeared to be strain-dependent, *Candida maltosa* can progressively adapt to the presence of bile salt by sub-culturing in gradually increasing concentration of bile salt (Noriega *et al*., 2004).

1.3 Objectives of this study:

The major objective of this study was to:

- Determine the probiotic potential of the isolate *Candida maltosa* species by using in-vitro analysis.
- Develop a probiotic supplement in baby food, using *C.maltosa* isolated from local mozzarella cheese.
- To check condition in different stress applying to the species.
- Acknowledge the existence as an applicable agent in probiotic, as this organism does not have any contribution in this probiotic filed.
- A study on *Candida maltosa* is not introduced and addressed adequately. To know more activities about this species, the experiment had been done on this.

Chapter 2

Materials and methods

2.1 Research location

This study had been carried out in the Industrial Microbiology Laboratory of the Institution of Food Science and Technology (IFST) division at the Bangladesh Council of Scientific and Industrial Research (BCSIR) located at Dr. Qudrat-I-Khuda Road, in Dhaka.

2.2 Materials

2.2.1 Equipment

- Laminar airflow cabinet
- Spectrophotometer
- Incubator and shaking incubator
- Vortex machine
- Autoclave machine
- Glassware, laboratory distillation apparatus- fractional distillatory set up, microscope, pH meter, petri-dishes, micro-pipettes, Bunsen burner, electric balance, conical flask, loop etc.

2.2.2 Isolates

- *Candida maltosa* from laboratory stock

2.2.3 Media

- **SDA (Sabouraud Dextrose Agar) and broth:**

It is used for the cultivation of yeasts, molds and aciduric bacteria from clinical and non-clinical samples. SDA is named after its inventor Carlier. This agar used for the cultivation of fungi, practically useful for the fungi associated with skin infections. This medium is also used in food, cosmetics and clinical specimens to assess microbial contamination. Mycological peptone contains nitrogenic compounds. The dextrose provides a source of energy. High concentration of dextrose and low pH promotes fungal growth and prevents bacteria from test samples being infected.

2.2.4 Reagents

- **PBS buffer:**

  A water-based salt solution containing disodium hydrogen phosphate, sodium chloride, potassium chloride, and potassium dihydrogen phosphate is abbreviated for Phosphate buffered saline. For most cells it is isotonic and non-toxic.

- **Bile salt:**

  Bile salt is a concentrated mixture of bile salts, containing primarily cholate of sodium and desoxycholate of sodium. Its primary use is as a selective agent for the isolation of gram-negative micro-organisms, inhibiting gram-positive cocci.

- **Gastric juice**:

  Artificial gastric juice was produced in the lab by using pepsin and water and pH was adjusted with HCl (Hydrochloric Acid).

2.3 Methods

2.3.1 Isolation and identification:

Test procedure:

Step 1: Culture isolation on Biolog recommended media:

- The cheese had been poured into the PBS (peptone buffer solution) for one day. The next day the cheese solution was taken and streak onto the media.
- Biolog recommended media (BUY) had been used and incubated at 26℃-28℃. All species that were identified, grew on this condition by the YT Microplate.

Step 2: Specimen preparation and characterization:

- Wet prep or gram staining was performed to verify yeast.
- The yeast was growing under the recommended condition. Specific selective media was implemented to grow yeast since it supports the growth and promotes retention of full metabolic activity to accurately match the metabolic patterns in the YT database.
- The yeast cells were freshly grown since many strains lose viability and metabolic activity in the stationary phase. The recommended incubation period for most of the organisms is 24 to 48 hours.
- Inoculation had been done more than one plate for insufficient growth for 24-48 hours.

Step 3: Inoculum preparation:

- Turbidity rage was established via turbidimeter.
- Turbidimeter was blanked with uninoculated water tube.
- A uniform suspension was prepared.
- Inoculum density was adjusted.
- The cell suspension was inoculated.

Step 4: Inoculation of the MicroPlate

- Microplate was labeled with the organism's name/number.
- The cell suspension was poured into the multichannel pipet reservoir.
- 8 sterile tips were securely fastened onto the 8-channel repeating pipettor.
- Tips were checked thoroughly.
- Wells were filled with 100 µl of chemicals.

Step 5: Incubation

- Microplate was incubated at 26℃-28℃.
- Moisture sources were provided.
- The plate was incubated for 24,48 or 72 hours.

2.3.2 Temperature test:

A temperature test was done for testing the highest survival temperature of the yeast species. Incubation temperature was the variable that influences the yeast in broth. 32℃-37℃ is the optimum temperature for *Candida maltosa*. Optimum temperature leads yeast to its fastest metabolism as well as resulting in the growth rate.

2.3.3 pH test:

Yeast can grow on acidic conditions properly; it can survive in extreme conditions such as low pH. To observe how the organism, survives in acidic or basic conditions, the pH tolerance test was done.

2.3.4 Salinity test:

To study the effect of saline concentration on the growth of *Candida maltosa* organisms were cultured in different saline concentrations. The growth pattern was observed by measuring

absorbance at 600 nm using a spectrophotometer. Flask with distilled water was taken and salinity was gradually increased to (1%-7%) in different flask by adding Sodium Chloride (NaCl).

2.3.5 Heat stress analysis:

Cells respond to a variety of environmental stresses by the adaptation of specific strain which depends on heat stress. The optimum temperature range of yeast is 90°F-92°F(32°C-35°C). The cell of *Candida maltosa* was exposed to the effect of mild and lethal heat stress and their viability was observed. The cultivation temperature was increased from 30°C to 44°C. *Candida maltosa's* optimum growth temperature is 32.5°C and it can survive till 37°C.

2.3.6 Osmotic pressure activity test:

To observe the osmotic pressure tolerance via sucrose and dextrose sugar, this experiment was done. Cell growth was measured by the optical density estimated at 600 nm (OD600) and the enumeration of colony-forming units on the agar plates up to 300 minutes.

2.3.7 Gastric juice tolerance assay:

In vitro gastric juice was produced freshly with pepsin 3.5 ml and added NaCl 1.5 ml (Sodium Chloride) later. The pH of gastric juice was adjusted by using HCl (hydrochloric acid) according to the value of pH were 2,2.5,3,3.5,4 and 4.5 and was inoculated with a fresh culture of isolates and incubated for 24 hrs at 32.5°C.

2.3.8 Bile salt tolerance assay:

Yeast culture was activated by in liquid Sabouraud Dextrose Agar (SDA) and inoculated at 32.5°C for 24hr. After that, the yeast cell was mixed by a vortex mixture. Afterward, tubes contain SDB (Sabouraud Dextrose Broth) with yeast cells supplemented with different concentrations of bile salt (0.1%,0.5%,1%). The growth was determined after 1^{st}, 2^{nd} and 3^{rd} hours of incubation at 32.5°C by measuring the absorbance at 600 nm.

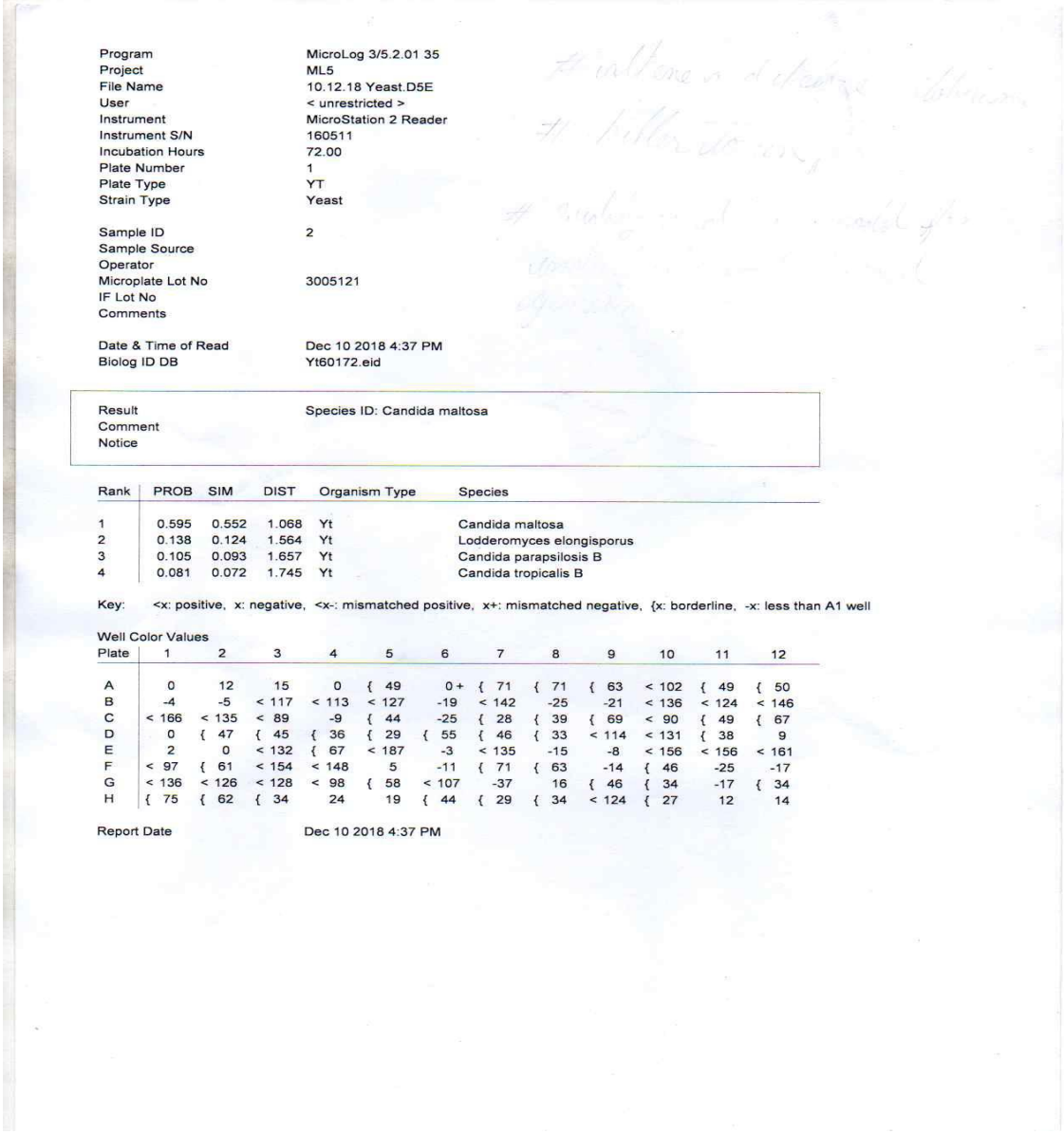
2.3.9 Statistical analysis:

All the experiment was carried out in triplicates. Data was presented as the mean +/- standard deviation (SD) for the indicated number of independently performed experiments.

Chapter 3
Results

3.1 Test procedure of isolation

THE BIOLOG Microlog™ 3 test: The YT MicroPlate™ test panel uses 94 biochemical tests to identify and characterize a wide range of yeast to provide a standardize micro method. MicroLog™ 3 software of Biolog is used in the YT MicroPlate to identify the yeast from its metabolic pattern. The selected strain was initially identified in this study based on physiological and biochemical characteristics and finally confirmed by the identification system of BioLog™.



Program	MicroLog 3/5.2.01 35
Project	ML5
File Name	10.12.18 Yeast.D5E
User	< unrestricted >
Instrument	MicroStation 2 Reader
Instrument S/N	160511
Incubation Hours	72.00
Plate Number	1
Plate Type	YT
Strain Type	Yeast
Sample ID	2
Sample Source	
Operator	
Microplate Lot No	3005121
IF Lot No	
Comments	
Date & Time of Read	Dec 10 2018 4:37 PM
Biolog ID DB	Yt60172.eid

Result	Species ID: Candida maltosa
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.595	0.552	1.068	Yt	Candida maltosa
2	0.138	0.124	1.564	Yt	Lodderomyces elongisporus
3	0.105	0.093	1.657	Yt	Candida parapsilosis B
4	0.081	0.072	1.745	Yt	Candida tropicalis B

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	0	12	15	0	{ 49	0+	{ 71	{ 71	{ 63	< 102	{ 49	{ 50
B	-4	-5	< 117	< 113	< 127	-19	< 142	-25	-21	< 136	< 124	< 146
C	< 166	< 135	< 89	-9	{ 44	-25	{ 28	{ 39	{ 69	< 90	{ 49	{ 67
D	0	{ 47	{ 45	{ 36	{ 29	{ 55	{ 46	{ 33	< 114	< 131	{ 38	9
E	2	0	< 132	{ 67	< 187	-3	< 135	-15	-8	< 156	< 156	< 161
F	< 97	{ 61	< 154	< 148	5	-11	{ 71	{ 63	-14	{ 46	-25	-17
G	< 136	< 126	< 128	< 98	{ 58	< 107	-37	16	{ 46	{ 34	-17	{ 34
H	{ 75	{ 62	{ 34	24	19	{ 44	{ 29	{ 34	< 124	{ 27	12	14

Report Date Dec 10 2018 4:37 PM

Figure 3.1 Yeast strain *Candida maltosa* was identified via MicroLog™ 3 software.

3.2 Growth of *C.maltosa* on agar media :

Semi-white yeast colonies with butter like firmness had been observed on SDA agar plate. Colony shape and surface appearance were used for the confirmation.

Figure 3.2: Colonies on SDA agar media after 24 hours incubation

3.3 Optimal growth temperature test:

Unlike the other environmental condition, yeast (*Candida maltosa*) internal temperature matches that of their environment. Optimum temperature leads the yeast to its fastest metabolism as well as resulting in growth rate.

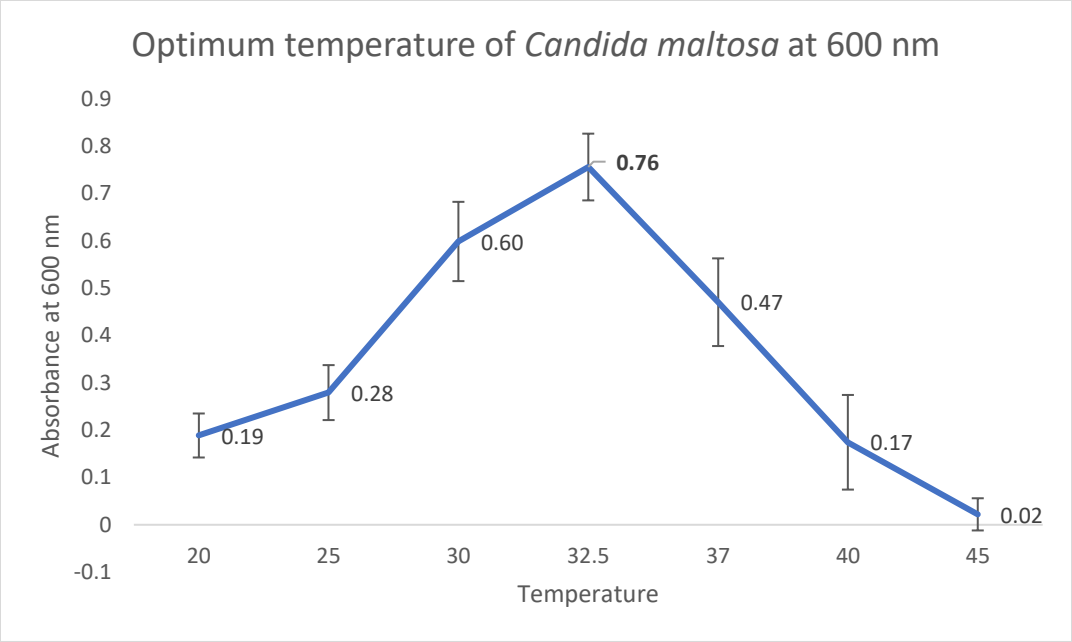


Figure3.3: Optimum growth of *Candida maltosa* at different temperatures.

Cell growth was monitored turbidimetrically by measuring the optical density at 600 nm using a spectrophotometer. Optimum growth temperature range was detected of the desired organism is 30℃-37℃. Between this temperature, *C.maltosa* grew well, which was the stationary phase of *C.maltosa*. At 25℃, *C.maltosa* started multiplying which was the log phase. At 30℃ the growth shows 0.60 and continues to grow at 37℃ where the absrobance is 0.47 . The optimal temperature was at 32.5℃ where the absorbance value (0.76) was the highest.

3.4 Optimal pH test of *Candida maltosa*:

Yeast can grow in acidic conditions properly; it can survive in extreme conditions such as at low pH. To observe how the organism, survives in acidic or basic condition the pH tolerance test was done.

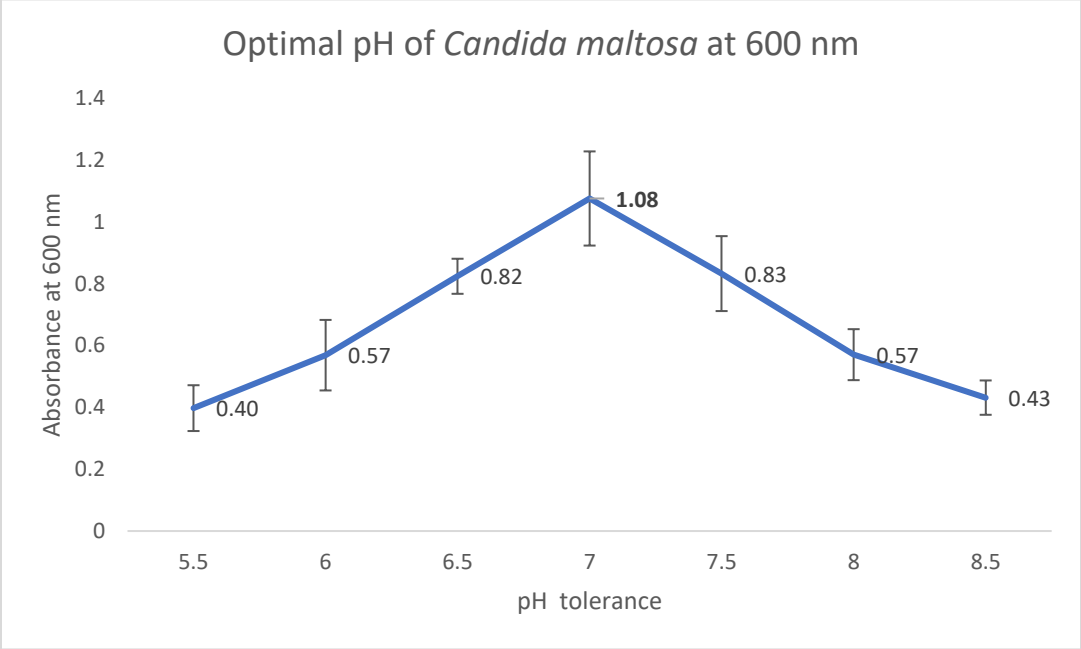


Figure3.4: Optimum pH absorbance of *C.maltosa* at different pH levels.

Figure 3.4. shows that the yeast species (*C.maltosa*) survived well at pH 7 it gave the highest peak (1.08) and the highest thrive, which is the neutral point of pH level. It declined from pH 7.5. So, from the figure, it can be said that *Candida maltosa* grew better at neutral pH, it could not tolerate the high acidic or alkaline range.

3.5 Salinity observation of *Candida maltosa*:

Sample organism was inoculated in SDB broth and incubated at 32.5℃ overnight. The flask containing media were autoclaved to be sterilized. To observe the salt tolerance capacity in isolates, different concentrations of salt had been produced

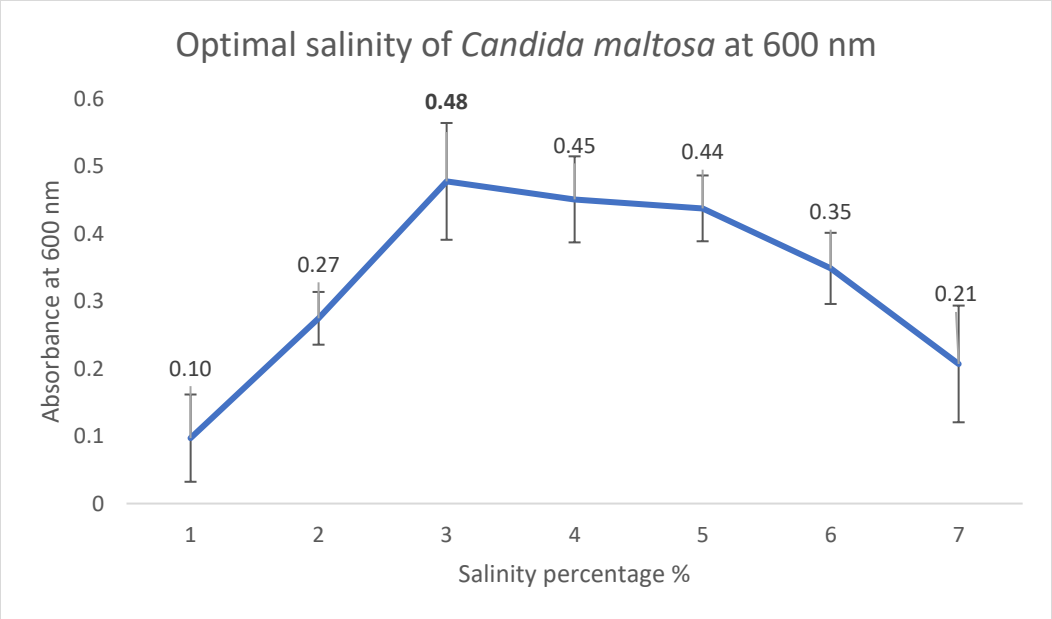


Figure 3.5: Salinity absorbance of *Candida maltosa* at different saline concentrations

Figure 3.5 shows that salt tolerance increasing gradually and at 3% concentration, the organism showed the highest growth where the absorbance value was 0.48 and the growth starts to decrease from 4% salt concentration.

3.6 Heat stress analysis:

Following the heat analysis, growth and OD had been observed. Heat stress was checked at 24 hours, 48 hours and 72-hours intervals.

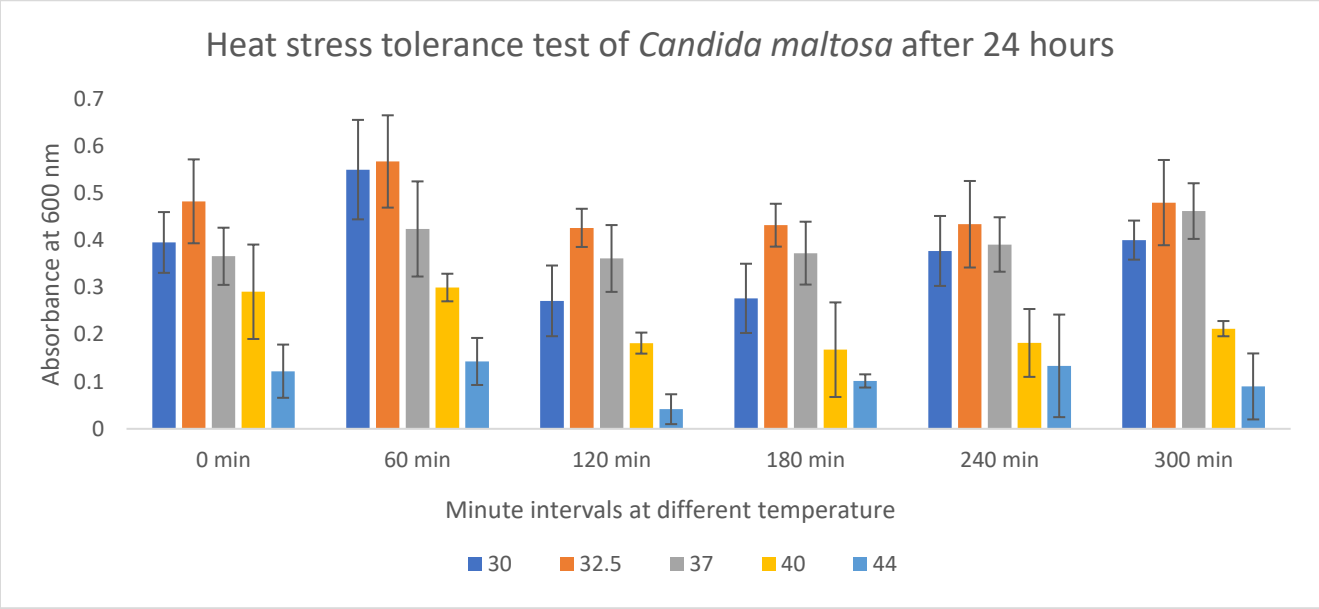


Figure3.6: Effect of heat stress at different temperatures at various minute intervals after incubation for 24 hours. Growth inhibition started at temperature 40℃ and above.

Table 3.1: CFU count of *Candida maltosa* after 24-hour incubation

Temperature (℃)	CFU (total colonies)
30	$1.6x10^5$
32.5	**$8.26x10^6$**
37	$4.0x10^4$
40	No colony
44	No colony

Table 3.1 showed the optimum growth of *C.maltosa* after 24-hours incubation at 32.5℃.

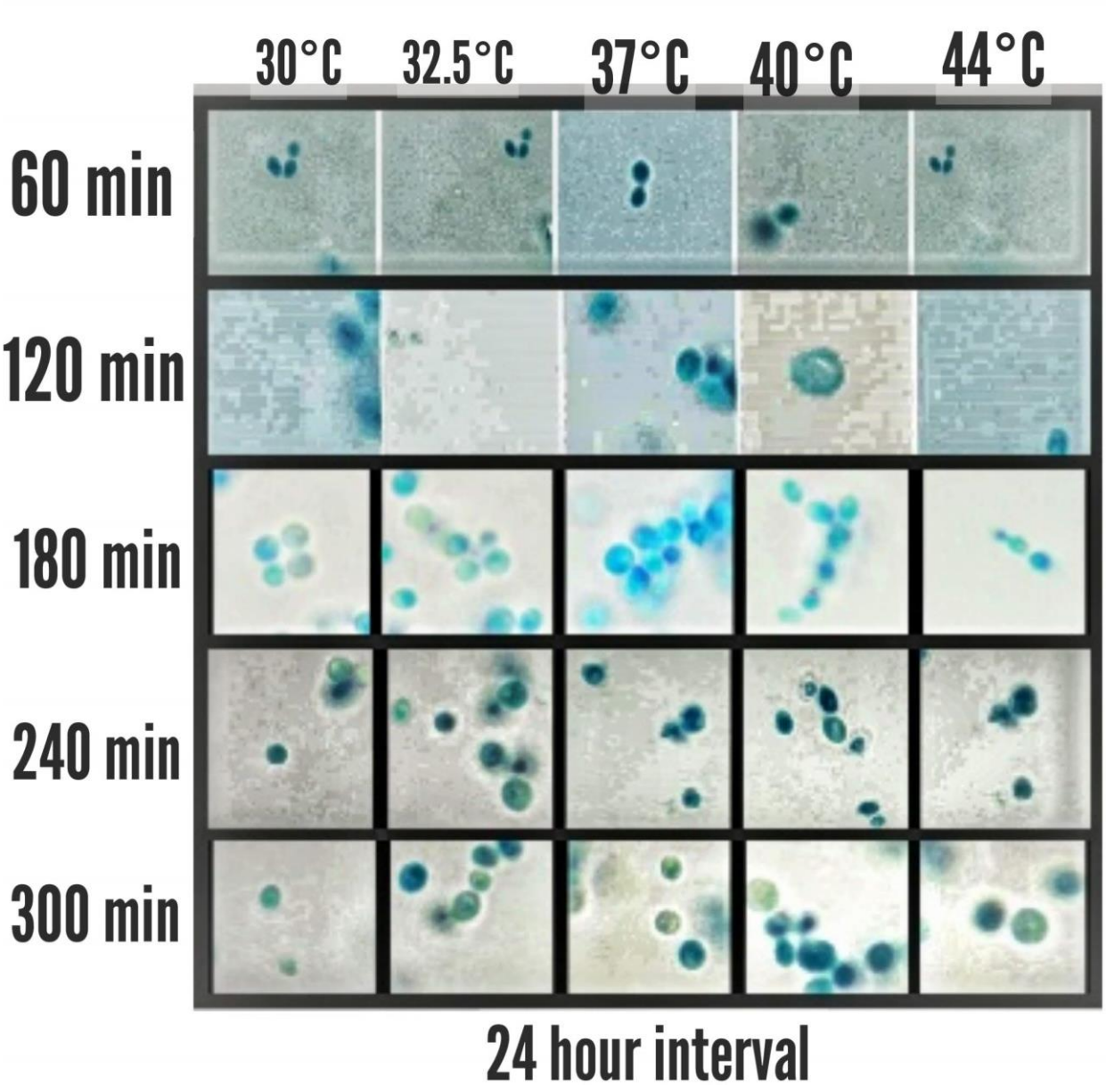


Figure 3.7: Morphological pattern at different temperatures at various minute intervals after incubation for 24 hours.

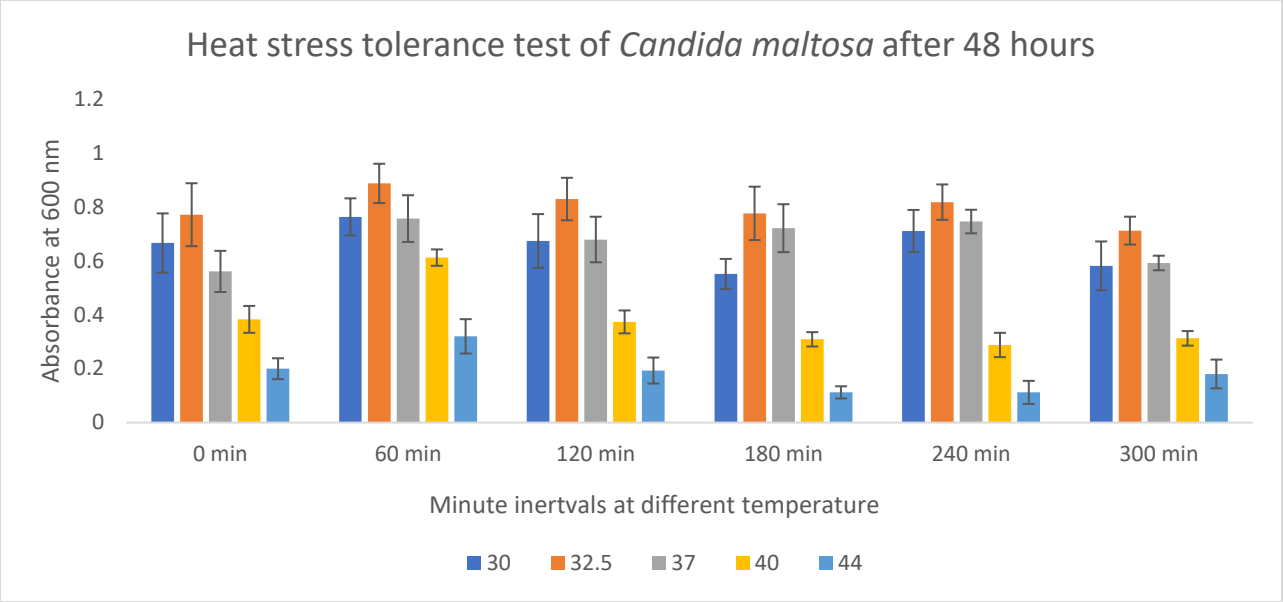


Figure 3.8: Effect of heat stress at different temperatures at various minute intervals after incubation for 48-hours. Growth inhibition started at temperature 40℃ and above.

Table 3.2: CFU count of *Candida maltosa* after 48-hours incubation

Temperature (℃)	CFU (Total colonies)
30	$2.0x10^5$
32.5	**$4.87x10^6$**
37	$1.0x10^6$
40	No Colony
44	No Colony

Table 3.2 showed the optimum growth of *C.maltosa* after 48-hours incubation at 32.5℃.

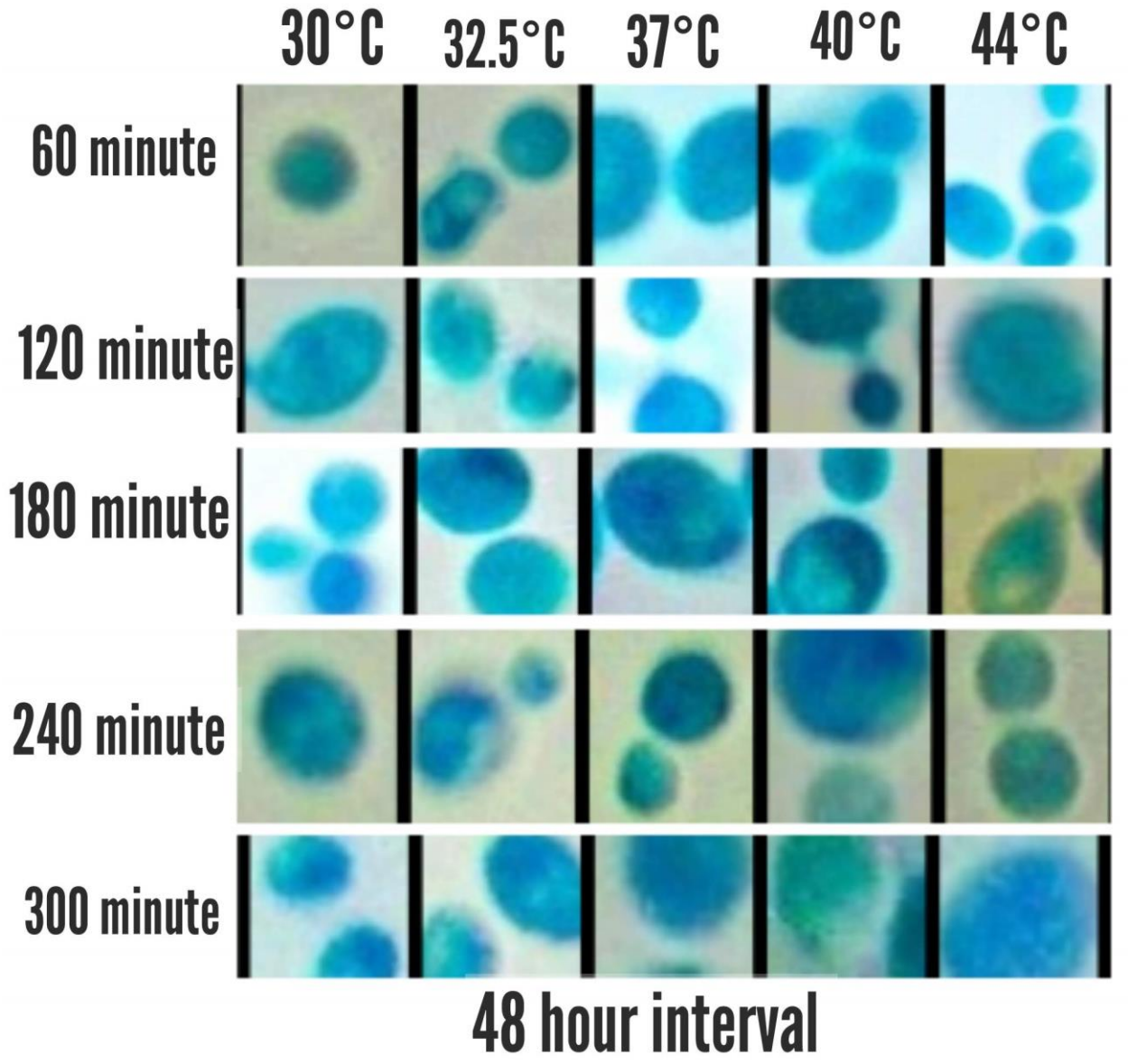


Figure 3.9: Morphological patterns at different temperatures and at various time intervals after 48 hours of incubation.

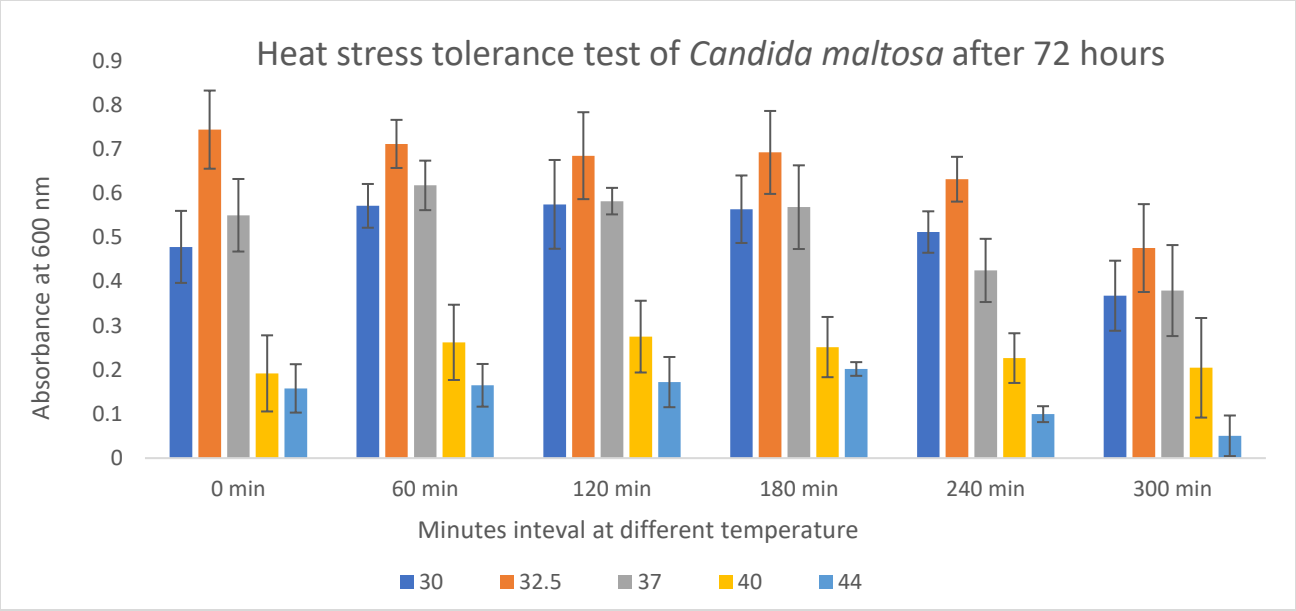


Figure 3.10: Effect of heat stress at different temperatures at various time intervals after incubation for 72-hours . Growth inhibition started at temperature 40° C and above.

Table 3.3: CFU count of *Candida maltosa* after 72-hours incubation

Temperature (°C)	CFU (Total colonies)
30	$5.5x10^5$
32.5	**$9.12x10^6$**
37	$3.4x10^5$
40	$2.4x10^5$
44	No Colony

Table 3.3 showed the optimal growth of *C.maltosa* after 72-hours incubation at 32.5°C.

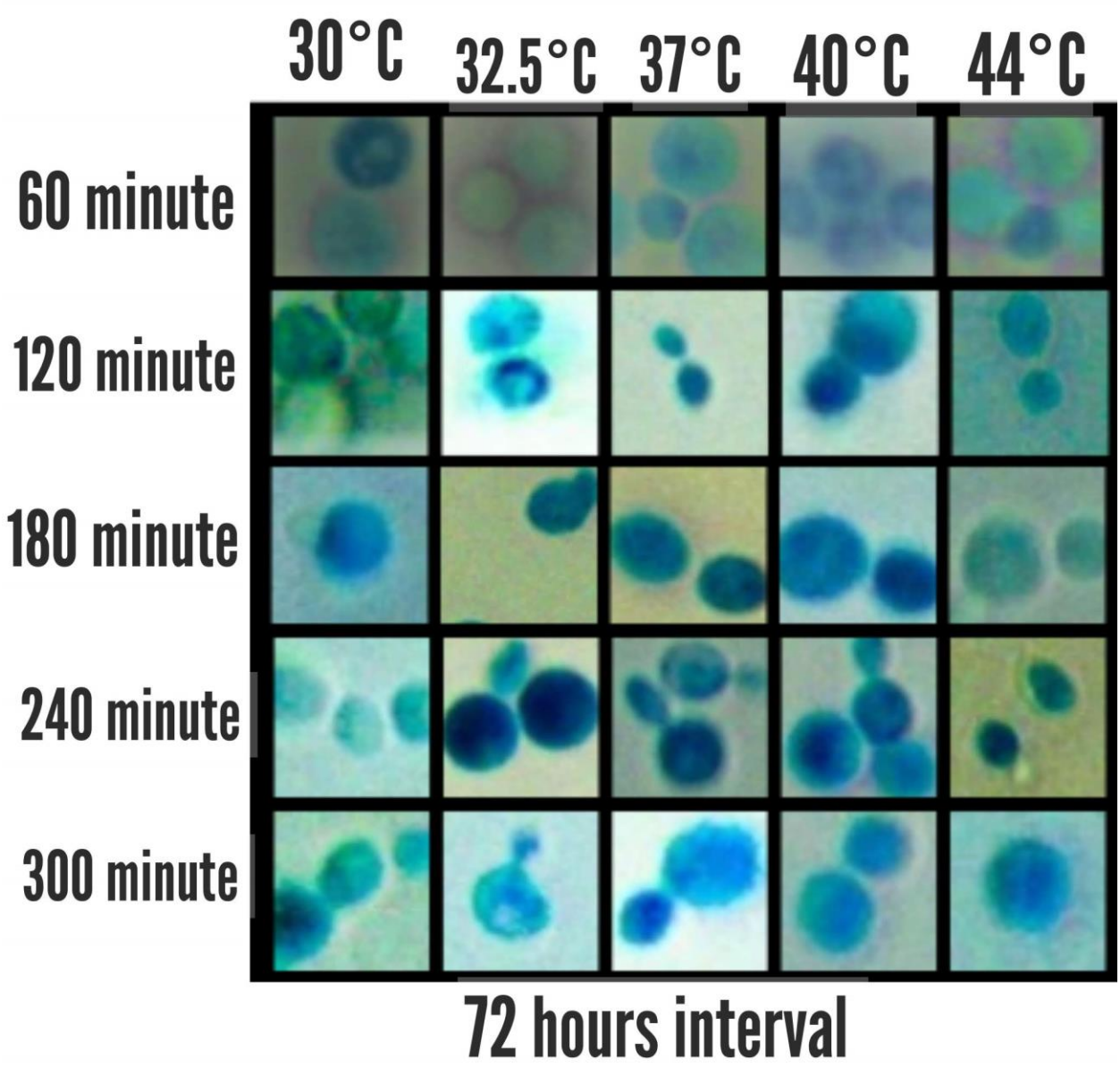


Figure 3.11: Morphological patterns at different temperatures at various minute intervals after incubation for 72-hours.

3.7 Osmotic pressure analysis of *Candida maltosa*:

To observe the osmotic effect on yeast cell growth, different concentrations of dextrose(0.04g/L, 0.12g/L, 02g/L, 0.28g/L, 0.36g/L) and sucrose (0.02g/L, 0.06g/L, 0.1g/L, 0.14g/L, 0.18g/L) was used. In this way, a constant force is maintained, driving this dextrose and sucrose water into the cell along its concentration gradient.

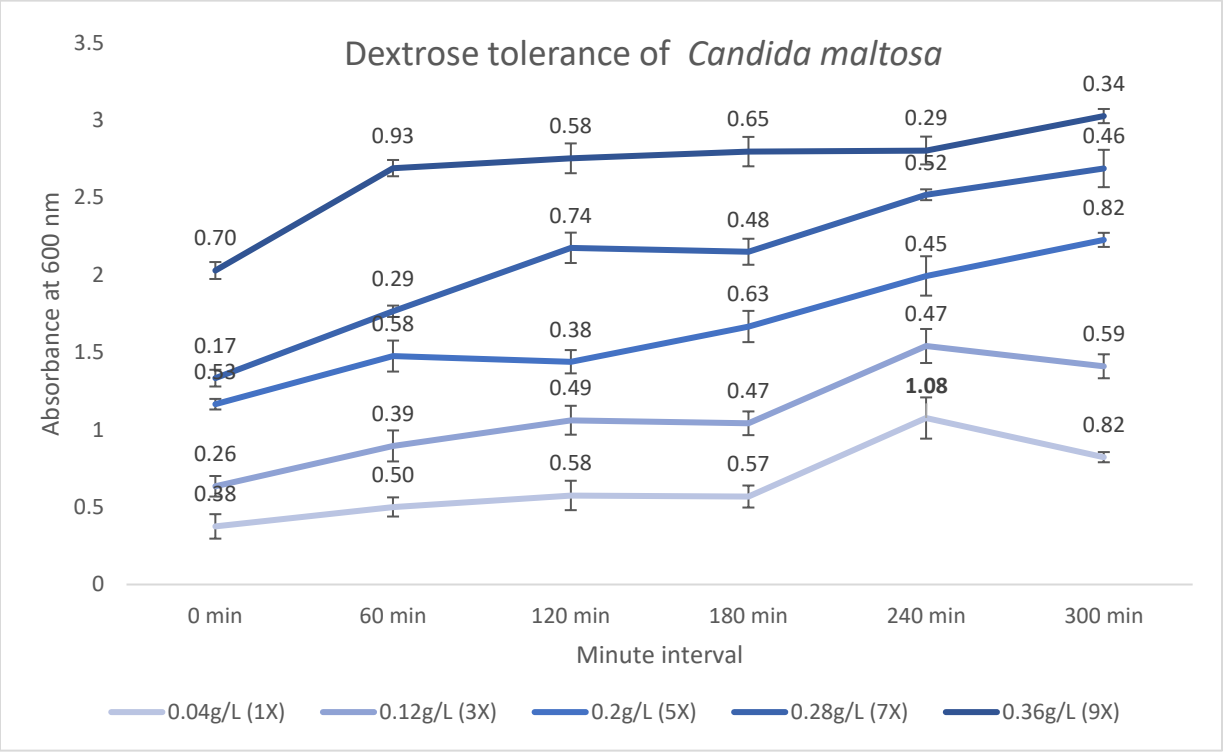


Figure 3.12: *Candida maltosa* in different dextrose concentrations at different minute intervals after 24 hours incubation at 32.5℃.

Reading was taken from exactly 24 hour incubation period which indicates 0 minute. The growth curve was going upward at 240 minutes in each concentration. This means the highest tolerance at 240 min. from above the result, it was said that in dextrose 1x (0.04g/L) concentrated solution *C.maltosa* gave the tolerance in 240 min, which was the highest thrive and tolerance, the absorbance value is 1.08. On the other hand, 7x (0.028g/L) dextrose concentration was the lowest tolerance with 0.17 absorbance value. So, in 60min, 1x concentration *C.maltosa* gave the highest tolerance**.**

Table 3.4: (CFU) of survived *Candida maltosa* at different dextrose concentrations.

Dextrose concentration (X)	0min CFU/ml	60min CFU/ml	120min CFU/ml	180min CFU/ml	240min CFU/ml	300min CFU/ml
1x	$3.09x10^6$	$4.36x10^6$	$1.84x10^6$	$3.36x10^6$	**$5.2x10^5$**	$1.0x10^6$
3x	$3.72x10^5$	$4.2x10^5$	$2.28x10^5$	$1.72x10^7$	$4.4x10^5$	$1.6x10^5$
5x	$6.0x10^4$	$1.2x10^6$	$1.62x10^6$	$2.23x10^6$	$3.9x10^5$	$4.36x10^7$
7x	$2.0x10^5$	$1.73x10^6$	$1.39x10^6$	$1.80x10^6$	$3.41x10^6$	$3.39x10^6$
9x	$5.6x10^5$	$2.32x10^6$	$4.75x10^7$	$5.04x10^7$	$4.92x10^7$	$1.09x10^6$

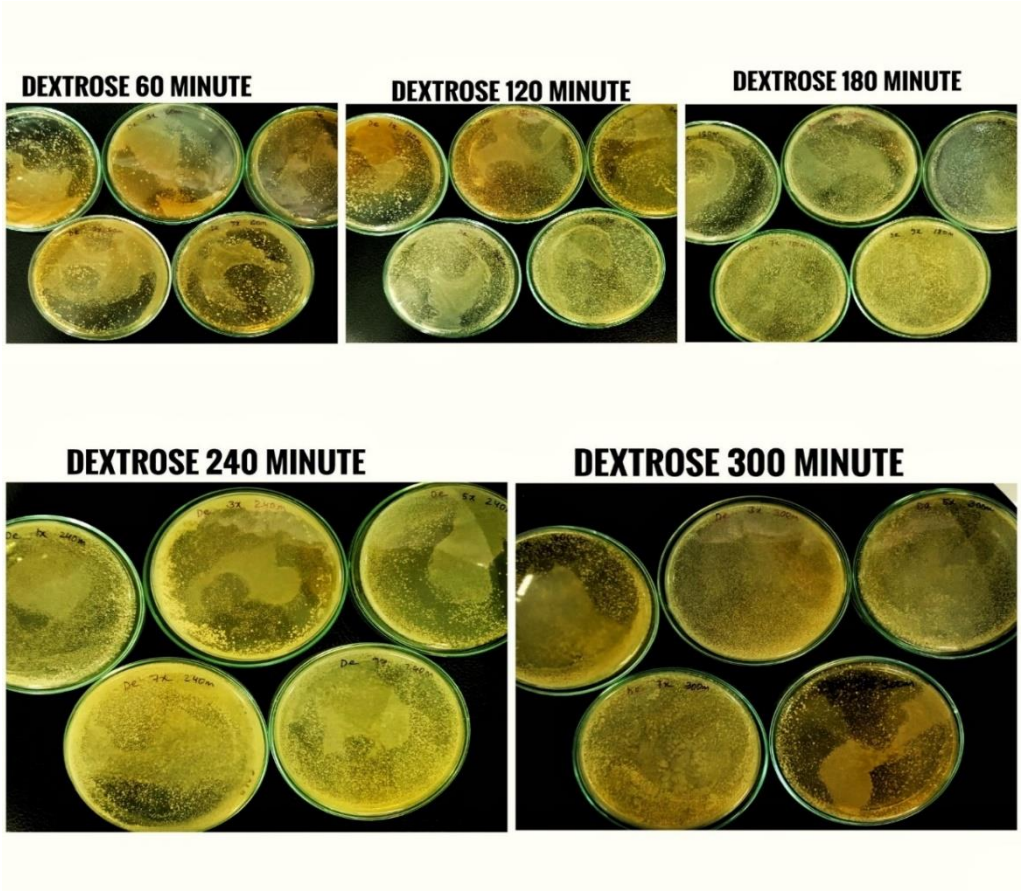


Figure 3.13: **Tolerance of *Candida maltosa* at different dextrose concentrations and different minute interval.**

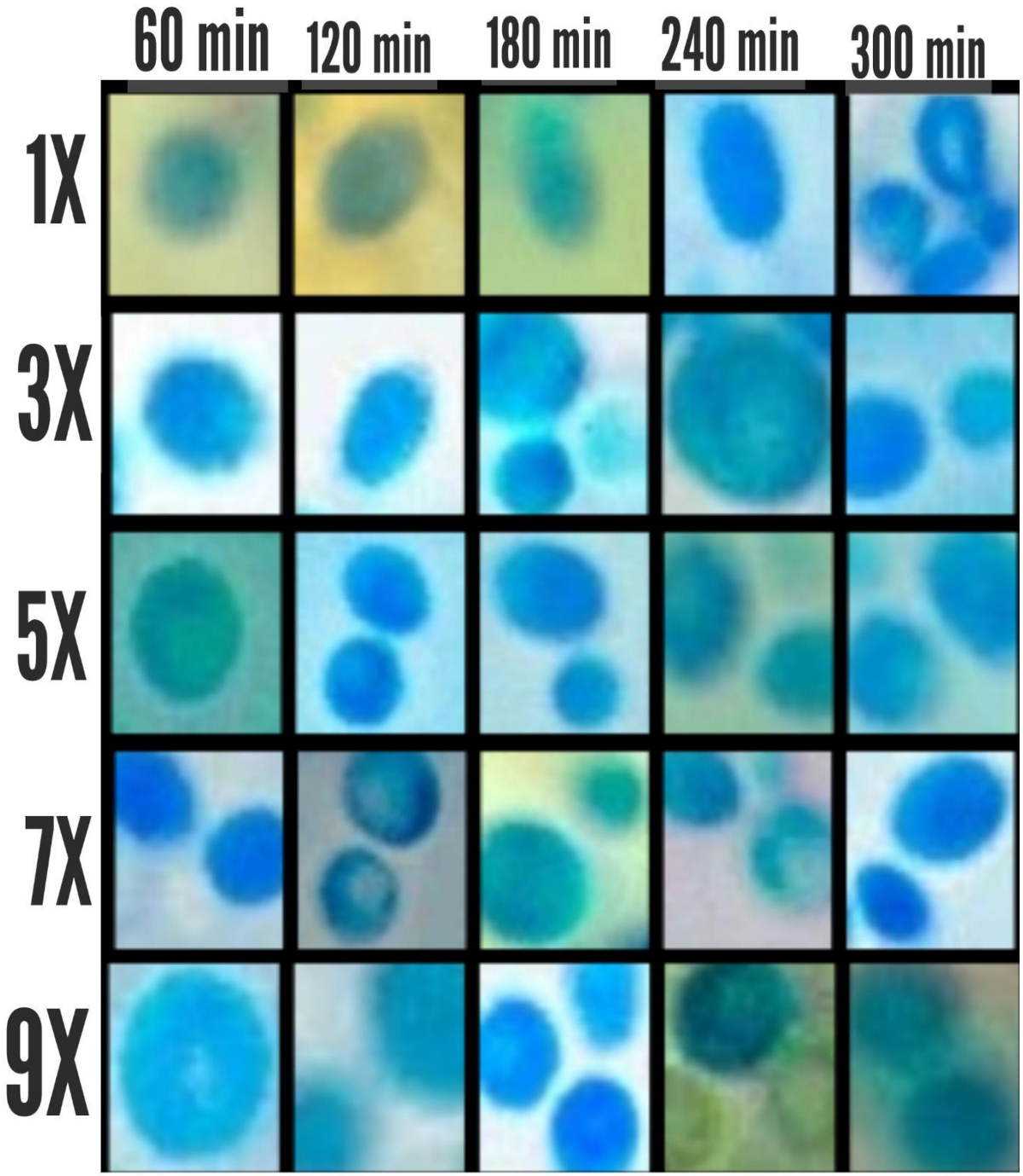


Figure 3.14: Morphological view of *Candida maltosa* at different dextrose concentrations at different minutes interval after 24-hours at 32.5°C incubation.

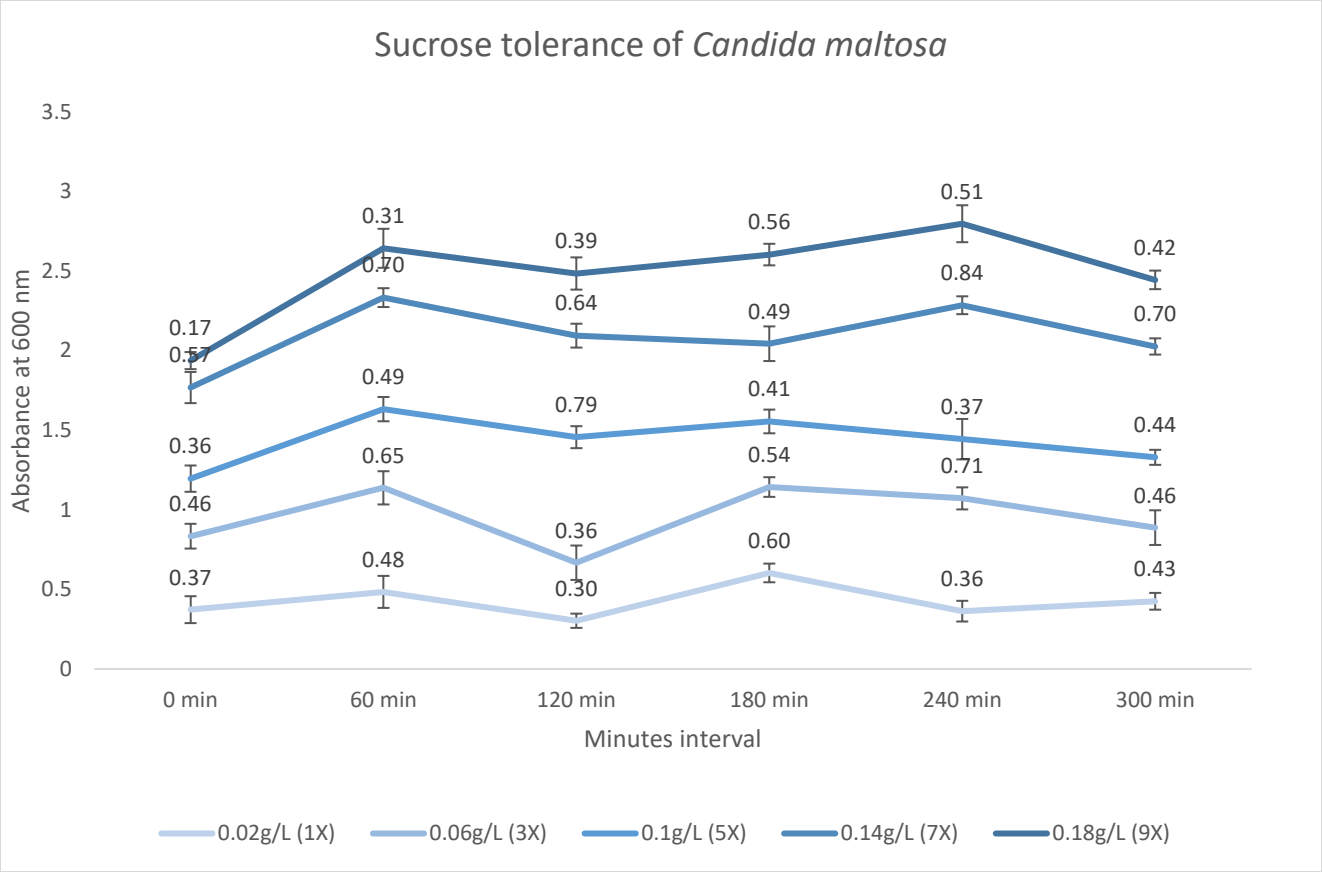


Figure 3.15: Tolerance of *C.maltosa* at different sucrose concentrations at different minute intervals after 24 hours incubation at 32.5°C.

Hence from the above result, in 7x(0.14g/L) sucrose concentration, in 240 minutes, the tolerance rate was the highest showing the value of 0.84. On the contrary, at 9x(0.18g/L) sucrose concentration, at 60minutes growth curve was less and the absorbance is 0.17, which indicate the less tolerance ability or the survival capacity of the organism at 9x, 60-minutes concentration.

Table 3.5: (CFU) of survived *Candida maltosa* in different sucrose concentration:

Sucrose concentration (X)	0min	60min	120min	180min	240min	300min
1x	$4.0x10^4$	$5.96x10^7$	$3.32x10^6$	$3.4x10^7$	$1.07x10^6$	$4.7x10^5$
3x	$2.2x10^5$	$2.88x10^6$	$2.48x10^6$	$1.92x10^6$	$2.7x10^5$	$2.33x10^6$
5x	$6.6x10^5$	$2.52x10^6$	$4.2x10^7$	$1.68x10^6$	$1.28x10^6$	$1.67x10^6$
7x	$1.34x10^5$	$2.12x10^6$	$2.24x10^6$	$1.63x10^6$	**$2.97x10^6$**	$1.03x10^6$
9x	$5.0x10^4$	$2.0x10^6$	$2.92x10^7$	$1.28x10^7$	$2.77x10^7$	$3.7x10^5$

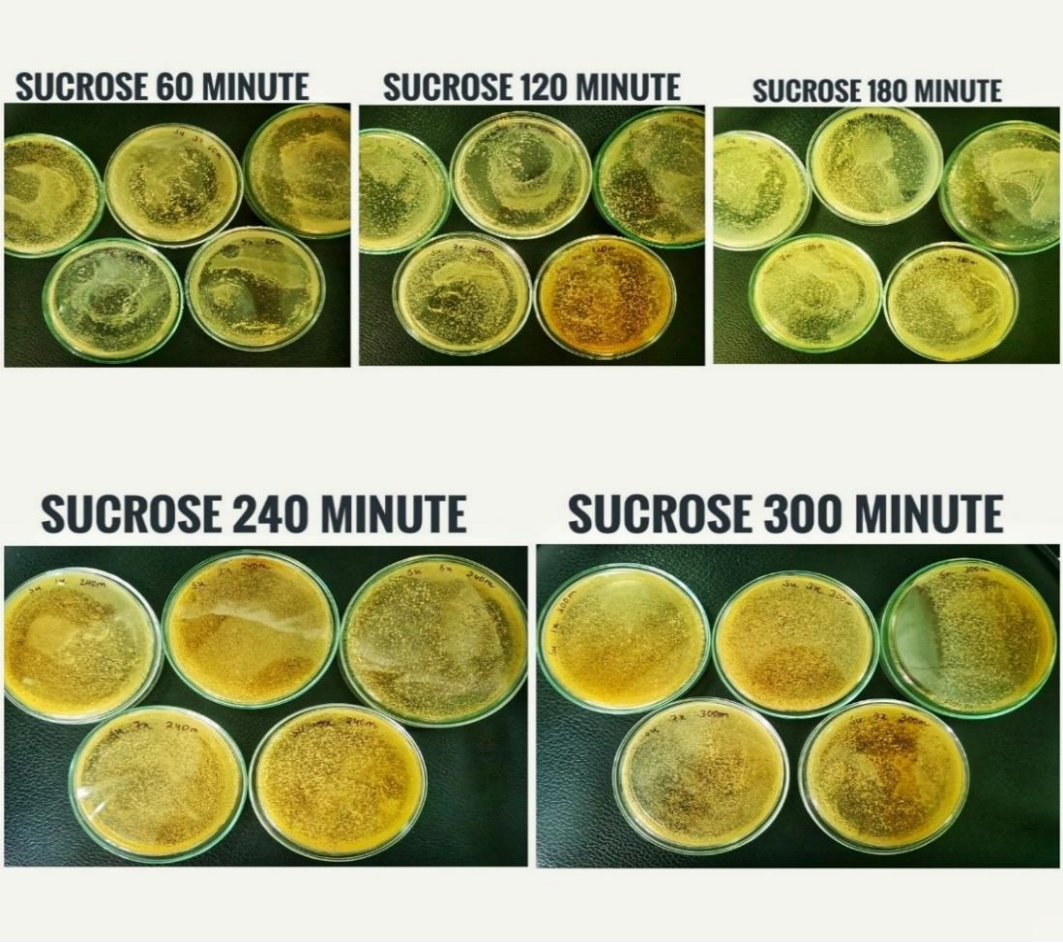


Figure 3.16: Tolerance of *Candida maltosa* at different sucrose concentrations and different minute intervals.

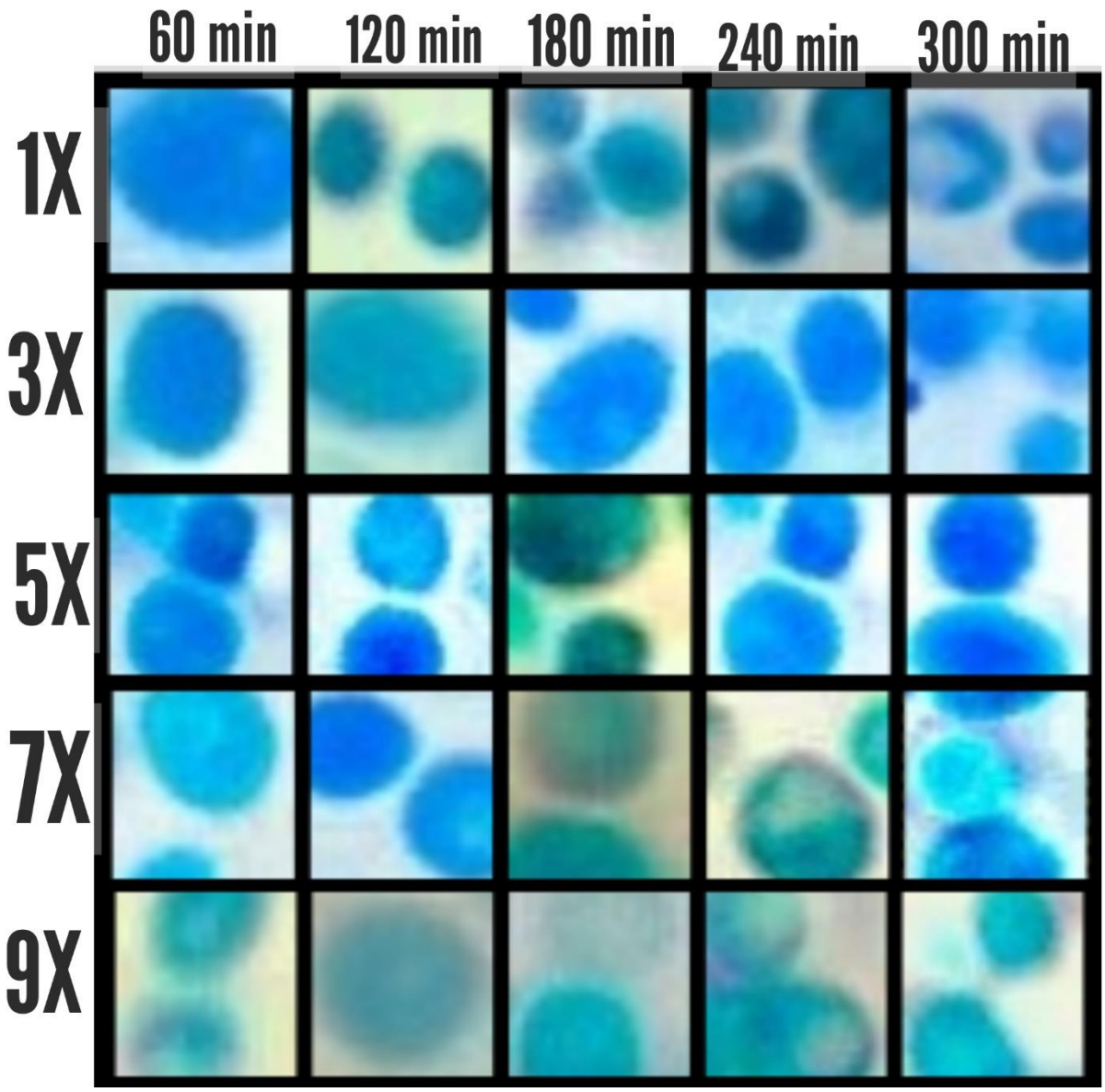


Figure 3.17: Morphological view of *Candida maltosa* at different sucrose concentrations and different minutes interval after 24-hours at 32.5℃ incubation.

3.8 Probiotic activity observation:

3.8.1 Gastric juice Tolerance Test of *Candida maltosa:*

The gastric juice tolerance of yeast was studied by modifying the pH of the SDB broth. The result had been checked 3 times in a 1hour time interval including CFU count of survived yeast cells in gastric juice.

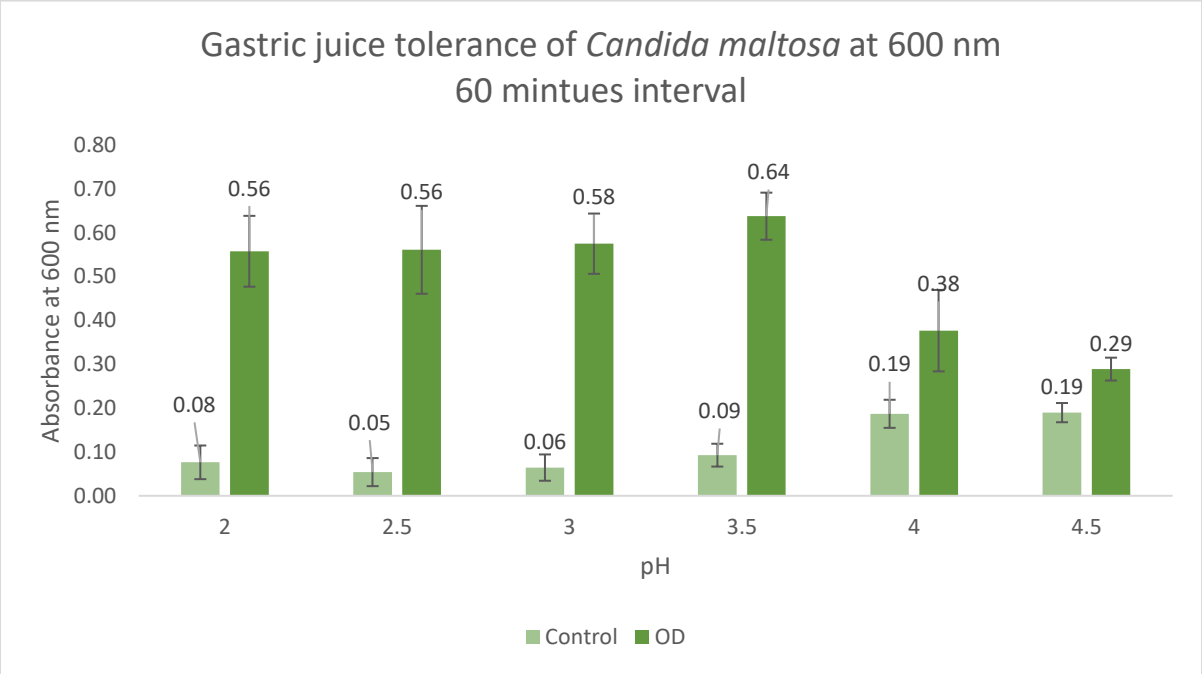


Figure 3.18: **The tolerance of *Candida maltosa* in gastric juice against control at 60 minutes interval. Figure indicated that the isolate can survive at pH 3.5 after 60 minutes of incubation at 32.5℃.**

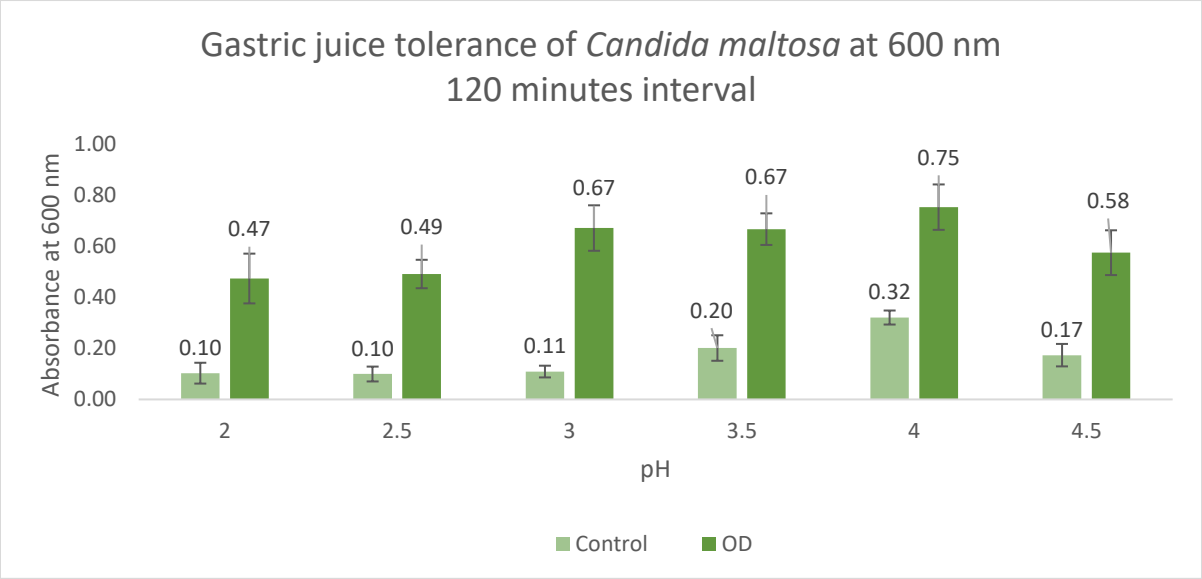


Figure 3.19: **The tolerance of gastric juice in *C.maltosa* against control in 120 minute interval. The isolated yeast can thrive up to pH 4 even after 120 minutes incubation period at 32.5℃.**

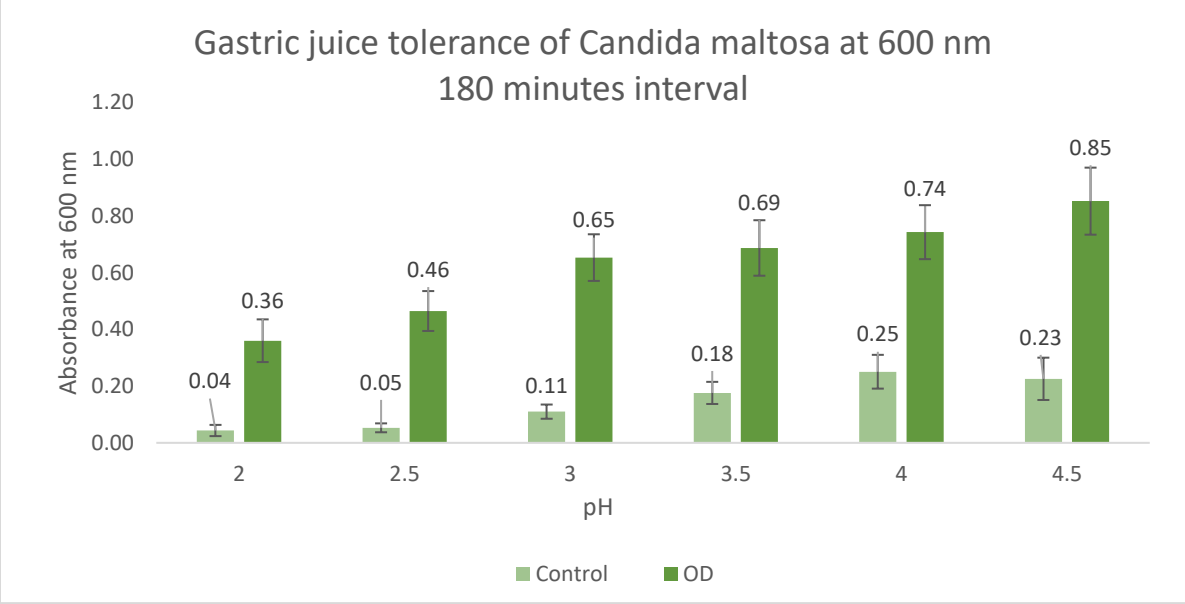


Figure 3.20: The tolerance of gastric juice in *C.maltosa* after 180minute interval. Figure showed that the isolated yeast can thrive up to 4.5 after 180 minutes incubation at 32.5℃.

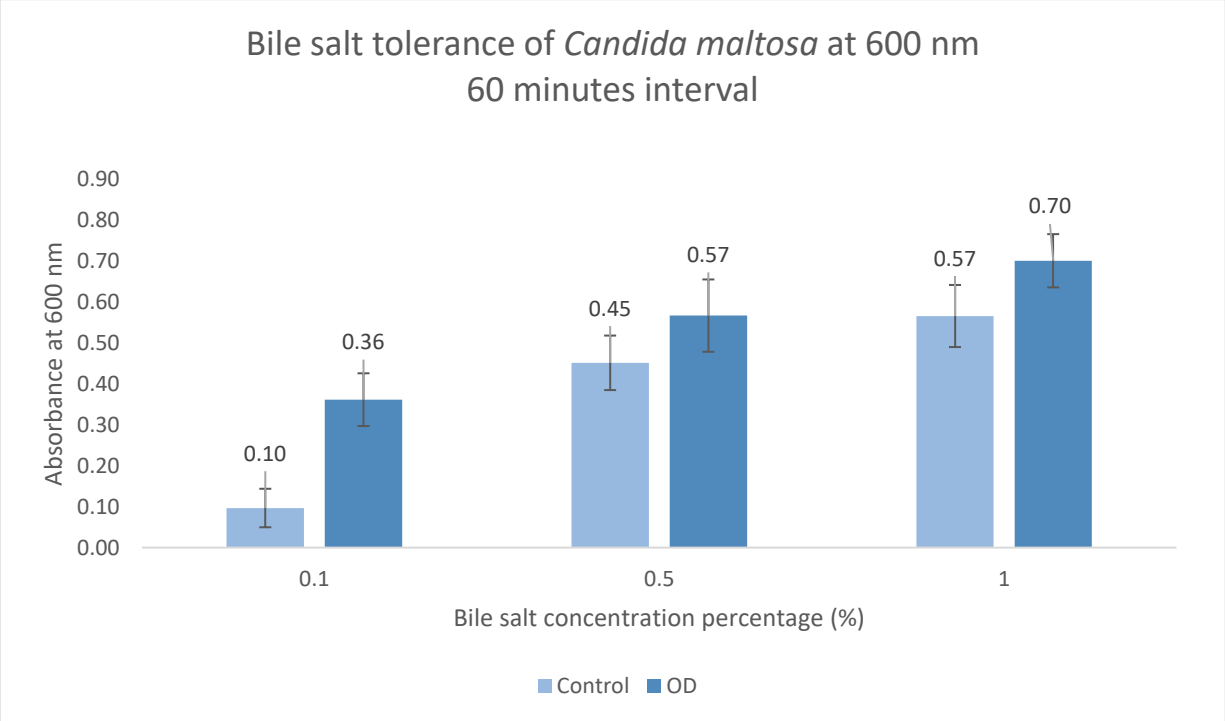
3.6 Table: the survival of *Candida maltosa* at 60, 120 and 180 minutes of gastric juice tolerance assay after 24-hours incubation at 32.5℃.

pH	Colony Forming Unit (CFU) 60 minutes	Colony Forming Unit (CFU) 120 minutes	Colony Forming Unit (CFU) 180 minutes
3	$3.7x10^5$	$2.1x10^5$	$3.1x10^5$
3.5	**$6.6x10^5$**	$4.2x10^5$	$5.7x10^5$
4	$6.0x10^4$	**$6.2x10^5$**	**$7.3x10^6$**

3.8.2 Bile salt tolerance assay test:

Freshly cultured organism was inoculated in bile salt at 3 different concentrations (0.1%,0.5%, and 1%). 10 ml of fresh SBD broth was inoculated with the organism and with or without bile (control), the culture was diluted up to 10^{-4} factor and consecutively pour plating was done and incubated at 32.5℃ overnight. Salt tolerance assay was checked 3 times at 1hours interval.

Figure 3.21: **Bile salt tolerance assay in *C.maltosa* after 60 minutes interval. At the highest percentage of bile salt, *C.maltosa* showed its tolerance after 60 minutes 24-hours incubation at 32.5℃.**



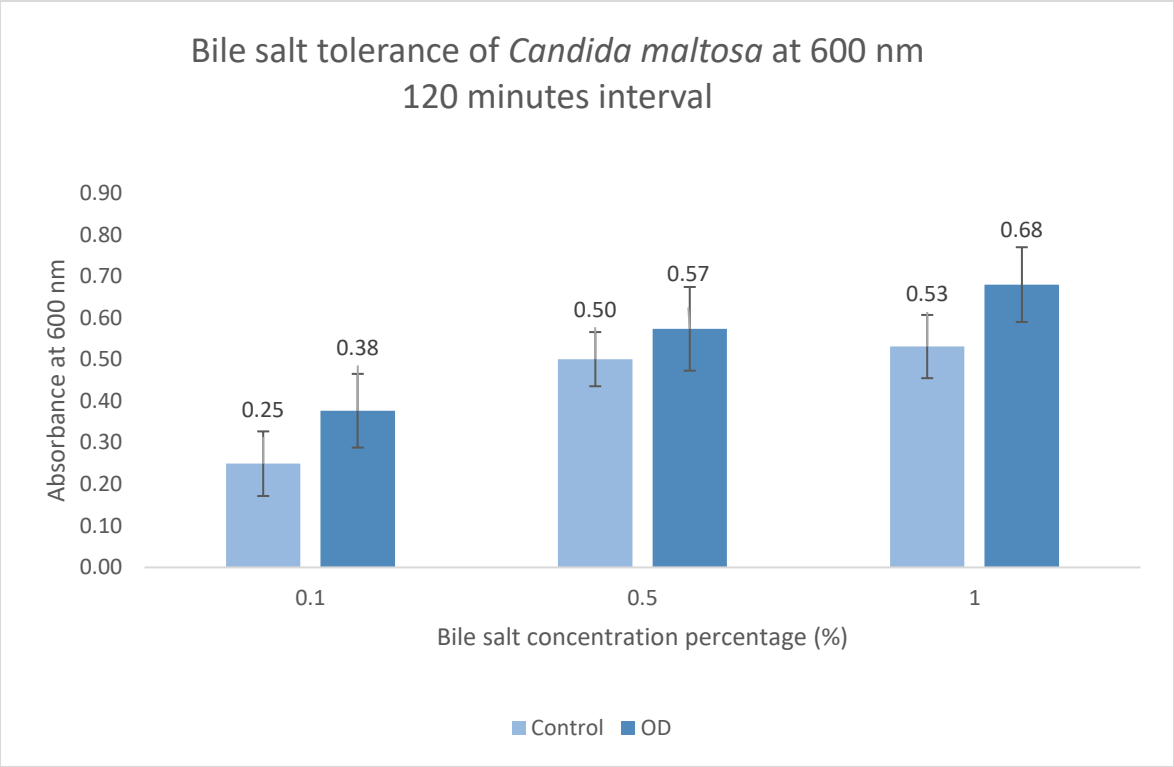


Figure 3.22: Bile salt tolerance assay in *C.maltosa* after 120 minute interval. Figure showed that, yeast can tolerate 1% bile salt after 120 minutes 24-hours incubation at 32.5°C.

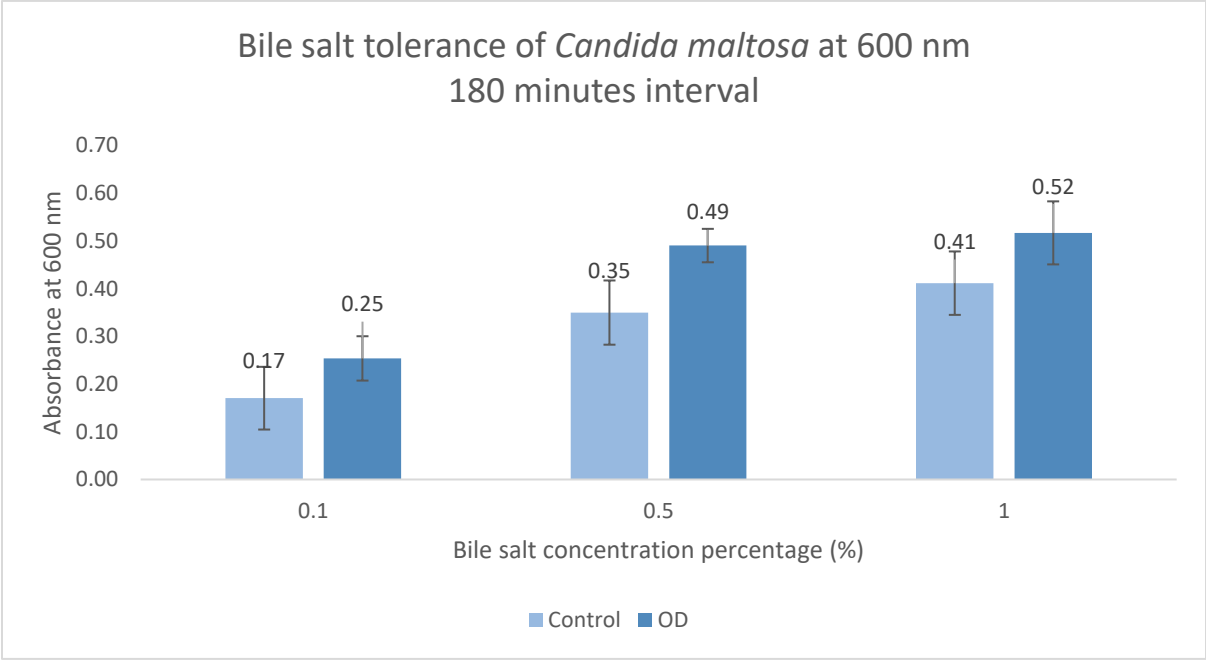


Figure 3.23: Bile salt tolerance assay of *C.maltosa* after 180 minute interval. figure, showed that, *C.maltosa* can thrive at 1% bile salt after, 180 minutes 24-hours incubation at 32.5°C.

3.7 Table: The survival of *C.maltosa* in CFU at 60, 120 and 180 minutes of bile salt tolerance assay after 24-hours incubation at 32.5℃.

Bile salt concentration (%)	CFU at 60 minutes	CFU at 120 minutes	CFU at 180 minutes
0.1%	$1.0x10^6$	$8.0x10^5$	$2.1x10^5$
0.5%	$3.52x10^6$	$4.0x10^6$	$3.01x10^6$
1%	**$4.2x10^5$**	**$3.1x10^5$**	**$5.2x10^5$**

Chapter 4
Discussion

Despite the availability of several industrial yeast strains, local isolates are usually altered more according to their own climatic condition. In this study, *Candida maltosa* was isolated form mozzarella cheese. The utilization of desired yeast in different analyses such as heat stress, osmotic pressure and probiotic activity check is an important strategy.

Based on the white and creamy appearance of desired yeast strain on solid media with oval cellular shape and BioLog read, it can be assumed that isolated yeast is the member of *Candida spp*.

Candida maltosa isolates from foreign cheese grew in both SDA (Sabouroud Dextrose Agar and Broth) figure (3.2) media adeptly but in other different media like Yeast Peptone Dextrose agar, Yeast Mannitol Agar, Rose Bengal Agar there was a certain amount of growth had been seen, which was not an adequate amount. Whereas other yeast species such as *Candida albicans* also grow in SDA, SDB beside in, PDA(Potato Dextrose Agar), YPD (Yeast Peptone Dextrose agar) as well (Binn Jatta, 2009). Recorded data indicated that *C.maltosa* can be identified as *Candida spp*. On top of that, a morphological view, the organism was found to belong to *Candida* type species.

Yeasts are activated in a very wide temperature range from 0 to 50℃, with an optimum temperature range of 20℃-30℃. *Candida maltosa* belongs to the mesophilic yeast which has an optimal growth temperature of around 32℃-34℃, but it continues to grow at 37℃, with growth up to 40℃-41℃ but in less amount, with the increasing temperature there is decreasing of yielding and protein content, however, the rate of decreasing depends on strain (Gradova *et al*., 1983). From the thermal tolerance test or temperature test analysis the data showed that *Candida maltosa* was grown best from 30℃-37℃ and optimum temperature was 32.5℃ (figure 3.3) as same as another strain of yeast called *Bacillus subtilis*, besides other Candida species like *Candida albicans* growth temperature around 33℃, maximum temperature is 38℃, On the other hand, *C.tropicalis*, thermotolerant yeast cannot grow at 43℃. In this extreme temperature, several species can survive,

whether the other Candida species like *C.tropicalis, C.krusei, C.glabrata* has optimum temperature 37℃ (Sathiya T *et al*., 2015). These strains cannot thrive at an extreme temperature of more than ~40℃.

In most micro-organisms, the optimum pH is above the neutral point (pH 7.0). Molds and yeasts are usually tolerant of acidity and are often associated with acidic food spoilage. Both yeast and mold can grow in an acidic and alkaline condition. Yeast can grow from 3.5 to 4.5 pH and molds grow from 2 to 8.5 pH, but prefer acidic pH (Mountney *et al.*, 1988) (Desrosier *et al*., 2018). *Candida maltosa* also can survive (figure 3.4) best in neutral position (pH 7) and it gave the optimal growth.

Many of the *Candida spp.* studied so far are osmotolerant and can grow with the high concentrations of salts and other osmolytes, as shown for *C.tropicalis* and *C.versatilis* (Sychrova *et al*., 2010). The effect of salt (NaCl) had been checked in different salt percentage (1%-7%). *C.albicans, C.parapsilosis* is the most salt tolerant in general. Desired yeast *Candida maltosa* gave the highest absorbance (figure 3.5) at 3% salt concentration, and then it started declining the tolerance phase.

A major study had been conducted in heat stress tolerance. Extreme temperature like 40℃-44℃ had been emphasized in this study. From the above result figure (3.6,3.8,3.10) it had been demonstrated that *C.maltosa* cannot tolerate the extreme temperature. It can reproduce at the higher temperatures, but the rate is too slow, compared to an optimal range of temperature e.g., 30℃-37℃. From 40℃-44℃ it gave growth to a smaller extent, because, with increasing temperature, the yielding and protein content decreas (Wolf, 1996) e, which had been seen through morphological overviews figure (3.7,3.9,3.11).

It was also observed the effect of elevated concentrations of sugar (dextrose & sucrose) figure (3.12 and 3.16) as another stress stimulant (Munna *et al.*, 2015). Cell growth was calculated by optical density estimated at 600 nm (OD600) and the enumeration of colony-forming units on the agar plates up to 300 min (Munna *et al*., 2015).

In this study, it was found out, that the desired yeast had the significant ability or tolerance in sucrose at 7x concentration. On the contrary, in dextrose, it showed less tolerance in 1x

concentration compares to sucrose. This means *C.maltosa* can tolerate a high concentration of sucrose sugar at an optimal temperature.

Finally, to find out some probiotic activity, two in-vitro tests were done. To check the probiotic activity gastric juice tolerance and bile salt tolerance test were performed. Probiotics are live micro-organism, which provided health benefits while consumed by improving or restoring gut flora.

By producing artificial gastric juice in the lab, the experiment had been conducted. Isolated yeast was used to examine the tolerance to gastric juice, and it showed the best activity. Probiotic yeast must survive transit through the stomach before reaching the intestinal tract and be subjected to gastric acid constituents, which is the primary mechanism of defense against most ingested micro-organism (Marteau *et al.,* 1993) it showed in figure(3.18, 3.19 & 3.20) that *Candida maltosa* has the ability to resist acidity, it survived at 3.5 pH satisfactorily, moreover, the organism had the ability to survive at high pH like 4.5 after 180 minutes, where it gradually cross the gastric juice pH (3.5) and survived. According to the study, it can be said that, *Candida maltosa* has proper gastric juice tolerance.

Bile tolerance is one of the most important properties for probiotic bacteria, as it determines the ability to survive in the small intestine and hence, the ability to function as a probiotic (Ruiz *et al.,* 2013). Following to the probiotic experiment, in bile salt, *C.maltosa* showed some acceptable tolerance. In 3 different concentrations of bile salt, (0.1%,0.5%,1%) *C.maltosa* showed its survival rate, at 3 hours interval. In figure (3.21,3.22,3.23) yeast was shown the best ability to tolerate bile salt and gave a good growth rate at 1% bile salt even after 180 minutes. Micro-organisms that exist in the acidic environment of the stomach must also survive in the intestinal secretion and in duodenum bile salt (Petaja *et al*., 2000). Bile resistance is an important criterion in culture selection as a dietary supplement (Walker *et al.,* 1990) because it could permit the growth of the ingested probiotic microorganism in the intestinal tract (Gilliland *et al*., 1984).

This finding may prove that, *Candida maltosa* may be recognize as a suitable probiotic in future. Different strain yeast such as, *Saccharomyces cerevisiae* established as agreeable probiotic, declared by World Medicine Organization, which has been used widely as low cost and efficient adjuvant for several gastrointestinal tract discorder such as, diarrhea. As *Candida maltosa* also

showed two positive results in probiotic test and followed the guide lines of probiotic, it is suggested to recognize as potential probiotic.

Conclusion

In conclusion, it can be stated that the microscopic observation and morphological views, yeast-like cells were recognized. As well as, the identification through BioLog software claimed that the species is *Candida maltosa.* Moreover, the temperature, pH and salinity test gave the almost same optimal range, like another *Candida spp.*

The isolate showed the optimal temperature of 32.5℃ with a pH of 7.0 as indicated by the absorbance. Moreover, the salinity range was high in 3%, which is the ideal salinity range of yeast.

Other experiments such as heat stress showed that the organism could not survive at extreme temperatures like 40℃-44℃ compared to the optimal range. Whereas, in osmotic pressure, *C.maltosa,* tolerate the highest pressure in sucrose rather than dextrose.

Furthermore, it can be said that the isolate showed good probiotic activities in both in-vitro analyses of gastric juice and bile salt tolerance. So, by observing these tests, it can be said that this isolate might be considered a good probiotic.

More probiotic in-vitro, as well as in-vivo test, should be run to produce effective probiotic supplements. However, an antifungal test should be experimented to check the activity against some pathogenic bacteria or yeast. If it is considered as a potential probiotic in near future, researchers should produce some value-added product from this yeast strain.

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Appendix

Laboratory instruments used throughout the study

Incubator	Memmert
Freeze	Siemens
Micro centrifuge	Mikro 120
Microscope	Olympus BX41
Weighthing balance	A&D Company
pH meter	Hanna Instruments
Laminer flow cabinate	ESCO
Micropipette	Eppendorf
Laboratory Glass bottles	Schott duran
Conical Flasks	Pyrex
Petri dish	Steriilin
Autoclave	Systec
Spectrophotometer	PG Instruments (T-60 UV visible)