INFLUENCE OF HEAT SHOCK AND OSMOTIC STRESSES ON THE GROWTH AND VIABILITY OF *Candida shehatae var shehatae* AND EXPLORE ITS PROBIOTIC POTENTIALITY

SUBMITTED BY

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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

The thesis/project titled “INFLUENCE OF HEAT SHOCK AND OSMOTIC STRESSES ON THE GROWTH AND VIABILITY OF *Candida shehatae var shehatae* AND EXPLORE ITS PROBIOTIC POTENTIALITY” submitted by Jannatul Sumaiya Promee Of 15th semester, [2020] has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in BIOTECHNOLOGY on [30-01-2020].

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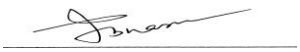

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Abstract

Live microorganisms with health benefits termed as probiotics, commercialized in the market to provide the health benefits to host. However, most probiotics are bacteria but very recently *Saccharomyces boulardii*, a yeast commercialized in the market. Yeasts also carry probiotic potentiality which is beneficial to our health but yet to be commercialized. The present study emphasizes how a yeast strain functions in critical growth temperature and under osmotic stress. This paper also analyzes the in vitro probiotic activity of the yeast strain. This study started with the isolation of yeast from a cheddar cheese sample. Identification was done by BIOLOG which is a very recent and easy microbial identification system. Isolated yeast *Candida shehatae var shehatae* showed its optimum growth in SDA (Sabouraud dextrose agar) medium at 32.5°C. To determine the critical growth temperature of *Candida shehatae var shehatae* growth was monitored at 40 °C and 45°C for 72 hours. Osmotic stress tolerance was observed at 32.5°C in different dextrose (0.04 g/l (1X), 0.12 g/l (3X), 0.2 g/l (5X), 0.28 g/l (7X), 0.36 g/l (9X)) and sucrose, i.e., 0.02 g/l (1X), 0.06 g/l (3X), 0.1 g/l (5X), 0.14 g/l (7X), and 0.18 g/l (9X) concentrations. The yeast strain found to be tolerant at high 45°C but reached its dormant phase after that. Gastric acid and bile salt tolerance test was done to find out the probiotic activity. Freshly prepared gastric juice at different pH levels and (0.1%, 0.55, 1%) bile salt was prepared and the result was monitored at 600 nm at different time intervals. The viability of strains was analyzed by determination of CFU/ml. *Candida shehatae var shehatae* showed high absorbance at pH 3 and 3.5 in terms of gastric juice tests. It also had shown the best ability to tolerate bile salts and had a good growth rate even at 1% bile salt. However, only two in vitro tests had done to find out the probiotic activity. Hence, more in vitro and in vivo tests should be done to establish the yeast strain as a good probiotic.

Keywords: probiotic; *Candida;* heat stress; osmotic pressure; BIOLOG; dextrose; sucrose; bile salt; gastric acid;

Dedicated to

My Beloved

Parents

Acknowledgement

Praise is to Allah the most compassionate the most merciful for giving me persistence and potency to take part and complete this research study.

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Sincerely,

Jannatul Sumaiya Promee

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Table of Contents

List of Tables

List of Figures

List of Acronyms

SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
YMA	Yeast mannitol agar
YEPD	Yeast extract peptone dextrose
Candida.sh	*Candida shehatae var shehatae*

Chapter 1

Introduction

1.1 Background

Over the last few years, a huge number of new food and food components have been improved in industrial sectors with the help of various valuable microorganisms that are good for health and can be consumed. Many are launched in the market, attracting the interest and attention of consumers due to their medical and health benefits (Pennacchia, et al., 2008).

Yeast is a single-cell fungus with a diverse phylogenetic grouping. Yeasts are eukaryotes as a member of kingdom fungi (Kurtzman & Fell) that digest their energy sources externally and absorb the nutrient into cellular molecules. Budding is the asexual reproduction method of yeast and binary fission in some cases. Classification of a yeast are mainly based on the microscopic appearance of the cells, the mode of sexual reproduction, certain physiological features (especially metabolic capabilities and nutritional requirements) and biochemical features. Generally yeast cells appear colorless, but on artificial solid media they produce colonies that may be white, cream-colored, or tinged with brownish pigments. Colony characteristics are useful in the taxonomy of yeasts. Physiological characteristics are also used to a great extent in determining yeast species.

The concept of probiotics (which means, "for life") was introduced in the early 20th century by Elie Metschnikoff, it, however, gained momentum only recently with considerable and significant advances in the functional and health food market across the world. Probiotics are 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). Probiotics are non-pathogenic microorganisms that, when ingested in adequate amounts, exert a positive influence on their hosts' health (Nagyzbekky, et al., 2016). The growth properties and physiological capabilities of microbes often emphasize by microbiology. Microbes must overcome various stresses that suppress their ability to grow or their basic survival (Haruta & Kanno, 2015). Numerous strategies exist in bacteria to cope with stressful conditions including the formation of cysts and spores, changes in cellular membranes, expression of repair enzymes for damage, synthesis of molecules for relieving stresses, and so forth. The study was conducted to find out whether yeast has the same capability as bacteria to cope with stressful conditions including morphological change and others. Continuous stress is a selective pressure and may induce genetic modification (Haruta & Kanno, 2015).

Microbes in a senescent state do not seem to cease all metabolic activity, but rather they keep partially metabolically active to maintain viability and protect against stress conditions. Such

reduced metabolism, however, still necessitates appropriate amounts of energy. Various approaches are conducted to estimate the nominal maintenance energy in stable and nutrient-limited environments.

Microorganisms play a crucial role within the food industry. Yeasts have the ability to ferment sugars to ethanol and carbon-dioxide and hence they are extensively in the food industry. The most commonly used yeast, the baker's yeast is grown industrially. *Saccharomyces carlsbergensis* is most commonly used in the fermentation of most beers. The other yeast strains of importance are *Brettanomyces, Schizosaccharomyce, Candida, Cryptococcus, Debaryomyces, Zygosaccharomyces, Hanseniaspora, Saccharomyces.* (Rajshree, et al., 2015)

Probiotics are live microorganisms that can provide health benefits when consumed. These benefits are thought to result from the ability of probiotics to restore the natural balance of gut bacteria. Probiotics are usually found in fermented foods or taken as supplements (Hatoum, et al., 2012). What's more, they appear to be safe for most people. A promising candidate for the replacement of antibiotic growth promoters could be probiotics. The term probiotic is composed of the Latin preposition 'Pro' meaning for or in support and the Greek 'biotic' from the noun bios meaning life hence the term probiotic meaning 'for life' or 'in support of life".

1.2 Literature review

Many studies have been carried out over the years to isolate the various strains from various species and study its various physiology and biochemical properties. This literature review will focus on certain characteristics of *Candida shehatae* var *shehatae* such as its isolation, characterization, its probiotic potential, and survivability at environmental stress.

1.2.1 Isolation and identification

Isolation is often a long-drawn-out process since it requires repeated sub culturing to obtain a pure single culture. This study is aimed to research the isolation and identification of the strain from the cheddar cheese sample.

The identification process has conducted by BIOLOG system software.

1.2.2 BIOLOG

BIOLOG's advanced phenotypic technology provides valuable information on the properties of strains, in addition to a species-level identification. Molecular methods such as 16s

sequencing and MALDI-TOF provide no information about the properties of the strain. BIOLOG's carbon source utilization technology identifies environmental and pathogenic microorganisms by producing a characteristic pattern or "metabolic fingerprint" from discrete test reactions performed within a 96 well microplate. Culture suspensions are tested with a panel of pre-selected assays, then incubated, read and compared to our extensive databases. 96 well MicroPlates incorporate a patented Redox tetrazolium dye that changes color as a result of cellular respiration providing a metabolic fingerprint (Biolog.com, 2020).

The YT MicroPlate™ test panel provides a standardized micro method using 94 biochemical tests to identify/characterize a broad range of yeasts. Biolog's MicroLog™ 3 software is used to identify the yeast from its metabolic pattern in the YT MicroPlate (Biolog.com, 2020).

1.2.3 The Response of *Candida* to salinity stress

Salinity stress is a factor where it is measured that up to which concentration the organism can survive and tolerate. Salinity stress mainly observes in plants. In this study, various concentration has been given to find out the tolerance level of *Candida shehatae var shehatae*.

1.2.4 The pH tolerance of *Candida*

In order to find out the suitable condition for the organism that whether it's acidic or basic the optimum pH level is needed. The optimum pH level means the pH where it (enzyme) is most active. Enzymes can be thought of as biological catalysts that control many physiology related chemical reactions in the body of an organism. An enzyme can work most with efficiency at or close to its optimum pH. The optimum pH for many micro-organisms is close to the neutral point (pH 7.0).Molds and yeasts are sometimes acid-tolerant and are thus related to the spoilage of acidic foods. Yeasts can grow in a pH range of 4 to 4.5 and molds can grow from pH 2 to 8.5, but favor an acid pH (Battcock & Azam-Ali, 1998).

1.2.5 Optimum growth temperature

Optimum temperature is the temperature at which any operation, such as the culture of any special microorganism, is best carried on. According to the BrewMart website, the optimum temperature for yeast growth is 32 degrees C. Yeast cell death occurs above 38 degrees C.

1.2.6 Heat stress and Osmotic stress tolerance

The definition of heat stress is a situation where too much heat is absorbed by a person, a plant or an animal and causes stress, illness or even death. Heat stress is manifested by elevated body

temperature, hot, dry skin, and lack of sweating and neurological symptoms such as paralysis, headache vertigo, and unconsciousness. It can even responsible for heat cramps, heat hyperpyrexia and warmth stroke which can cause death.

Osmotic shock or diffusion stress is physiological disfunction caused by an unexpected modification within the substance concentration around a cell that causes a speedy modification within the movement of water. Osmotic stress may be a potent regulator of the conventional perform of cells that area unit exposed to osmotically active environments below physiological or pathologic conditions.

Abrupt changes within the environmental and chemical science stimuli together with temperature, pH, sugar/salt concentrations, the oxidation-reduction state, cyanogenic compounds, and nutrient exhaustion are largely found to elicit battery of defending response by up-regulating the genes secret writing heat shock proteins (HSPs) in microorganism cells. It can also cause heat cramps, heat exhaustion and heat stroke which may lead to death. Like bacteria, the heat shock response in *Candida*, the model experimental yeast species, has been also characterized by the rapid changes in their cellular physiology including the budding manner accompanied by the increased tolerance against elevated salt and sugar concentrations. Another study conducted where in *S. cerevisiae*, heat-sensitivity is ordinarily prescriptive of defects in protein coding genes which are also essential for maintaining the cell viability. The coupling consequence of heat stress together with the osmotic shock has been found to influence cellular degeneration along with the retardation in cell division in yeast cells (Munna, et al., 2015)

1.2.7 Probiotic activity test of *Candida*

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". During the last decade, the use of probiotics for humans has received increasing attention as scientific evidence continues to accumulate on the properties, functionality, and beneficial effects of probiotic bacteria on humans. The look for more new probiotics is driven by the growing demand for probiotic functional food and beverages and dietary supplements due to rising levels of health consciousness and growing consumer awareness regarding gut health and the concept of preventive health care (Shokryazdan, et al., 2014). In order to find out the probiotic activity in vitro and in vivo experiments are needs to be carried out.

1.3 Objectives of the study

The main objectives of this study were to:

- Determine the optimum growth temperature and pH level of *Candida shehatae* var *shehatae*.
- Observe the viability of yeast cells at high temperature and osmotic pressure.
- Determine the probiotic potential of the isolated *Candida shehatae* var *shehatae* by using in vitro analysis.

Chapter 2

Materials and Methods

This study was carried out in Industrial Microbiology Laboratory of the Institute of Food Sciences and Technology (IFST) division at the Bangladesh Council of Scientific and Industrial Research (BCSIR).

2.1 Materials

2.1.1 Isolates

In this study, isolate that had been used were obtained from the Industrial Microbiology lab and were isolated from cheddar cheese and identified with BIOLOG and morphological tests.

2.1.2 Equipment

- Laminar airflow cabinet
- Incubator
- Spectrophotometer
- Vortex machine
- Autoclave machine
- Microscope
- pH meter, petri dishes, micro-pipettes, micro-burette, Bunsen burner, etc.

2.1.3 Media and Solutions

Rose Bengal agar: Rose Bengal Agar is a selective medium to detect and enumerate yeasts and molds in food samples.

Yeast mannitol agar: Yeast Mannitol Agar is used for cultivation, isolation, and enumeration of soil microorganisms like Rhizobium species.

Yeast extract mannitol agar: YEPD or yeast extract peptone dextrose, also often abbreviated as YPD, is a complete medium for yeast growth contains bacteriological peptone, yeast extract, glucose.

SDA (Sabouraud Dextrose Agar) and SDB (Sabouraud Dextrose broth)

Sabouraud Dextrose Agar (SDA) is used for the isolation, cultivation, and maintenance of non-pathogenic and pathogenic species of fungi and yeasts. SDA was formulated by Sabouraud in 1892 for culturing dermatophytes. The pH is adjusted to approximately 5.6 in order to enhance

the growth of fungi, especially dermatophytes, and to slightly inhibit bacterial growth in clinical specimens.

Peptone (Enzymatic Digest of Casein and Enzymatic Digest of Animal Tissue) provide the nitrogen and vitamin source required for organism growth in SDA. **Dextrose** is added as the energy and carbon source. **Agar** is the solidifying agent.

Chloramphenicol and/or **tetracycline** may be added as broad-spectrum antimicrobials to inhibit the growth of a wide range of gram-positive and gram-negative bacteria. **Gentamicin** is added to further inhibit the growth of gram-negative bacteria.

The neutral pH of the **Emmons** modification seems to enhance the growth of some pathogenic fungi, such as dermatophytes.

Yeasts will grow as creamy to white colonies. Molds will grow as filamentous colonies of various colors.

2.2 Methods

2.2.1 Sample collection and processing

Samples were collected from cheddar cheese. Then the samples were weighed in grams. Samples were then dispensed into a prepared 150 ml of peptone water contained in the conical flask. The suspension was shaken gently for homogenous mixture. Samples were subjected to 10-fold dilution and were spread plated onto Rose Bengal agar for yeast and mold count.

2.2.2 Incubation and selection

After 24 hours of incubation, the total number of colonies on the agar medium were counted. The colony characteristics were observed. Colonies were further selected for the identification test in **BIOLOG ™** system.

2.2.3 BIOLOG ™ test:

The BIOLOG YT Microplate™ is designed for the identification and characterization of a very wide range of Yeasts. Since the YT MicroPlate™ measures both metabolic reactions as well as turbidity growth to produce identifications, it provides superior capability for all types of yeast organisms. The Biolog System makes identifying yeast nearly as easy to identify as bacteria. The isolates were cultured on biology universal growth (BUG) agar medium and incubated at 26-28°C for 24-48 hours. All species that can be identified with the YT MicroPlate

will grow under these conditions. After cleaning the uninoculated water tube at 100% transmittance adjustment on turbidimeter. Then the desired turbidity 47% T determined (according to the protocol YT (Biolog Catalog #3415). Using the Biolog turbidimeter and 20 mm diameter tubes, this should give a transmittance level of about 47%T. After that, the turbidimeter placed blank with a clean tube containing uninoculated water. Because the tubes used are not optically uniform, they should be blanked individually and not rotated in the light path of the turbidimeter. A uniform suspension was prepared by removing cells from the agar plate with a sterile cotton swab so as not to carry over any nutrients from the agar medium into the suspension. When the meter needle goes toward the acceptable turbidity range is defined as by the turbidity standard plus or minus 2% transmittance. Then the inoculum density adjusted by adding more water. The cell suspension then inoculated into the MicroPlate promptly. After labeling the microplate with the organism number,the cell suspension poured into the multichannel pipet reservoir. Used 8-Channel Repeating Pipettor all wells filled with 100 µl. Then the microplates covered with the lid. The plate was then incubated at 26-28°C for 24, 48 or 72 hours (Biolog.com, 2020).

2.2.4 Temperature and Media test

Media

Rose Bengal agar, Yeast mannitol agar, YEPD agar, SDA were used to find out the suitable growth solid medium for the organism. Those agar medium were prepared and autoclaved. After autoclaved the media poured into the petri dish and from the stock culture the organism was collected with the loop and streaked on to the petri dish for the desired culture. YPD broth, SDB were used as a liquid medium for the subculture.

Temperature

Suitable selected solid and liquid media were used as optimum media for the organism. A Temperature test was done in order to find out the optimum growth temperature for this organism. The organism was sub-cultured in those two media and placed in a different incubator at 20-45°C for 24, 48 hours. The Growth pattern was observed by measuring absorbance at 600 nm in different time intervals using a spectrophotometer.

2.2.5 Salinity and pH test

In order to identify the effect of saline and suitable pH concentration on the growth of isolate, the organism was cultured at different saline and pH concentration. The Growth pattern was

observed by measuring absorbance at 600 nm at different time intervals using a spectrophotometer. The Organism was inoculated in SDB and incubated at 32°C. The salinity was gradually increased to 1%, 2%, 3%, 4%, 5%, and 6% to 7% in different flasks by adding Sodium Chloride (NaCl). The pH was also set from 5.5-8.5. The culture was inoculated in different salinity and pH solution and was incubated at 32°C for 24 hours. At the end of the incubation period, cell density was determined by measuring absorbance at 600 nm using a spectrophotometer.

2.2.6 Heat and osmotic stress tolerance

Heat stress tolerance of *Candida shehatae var shehatae* was observed. The Organism was freshly grown on SDA. Pre cultures were prepared by inoculating 5 ml SDB by a loopful of a colony from the freshly prepared yeast cultures plate, followed by incubation at 30, 32.5, 37, 40 and 45°C in static condition up to 72 hours. The optical density at 600 nm (OD_{600}) and the capability to form the colony forming units (CFUs) were monitored at the specific time intervals. To determine the critical growth temperature, growth was monitored at 40 and 45°C. For morphological observation, an aliquot of 5µl from each of the culture suspension was removed at 60 mins intervals (Munna, et al., 2015).

Osmotic stress tolerance was observed by following the same procedure as heat stress tolerance. Pre cultures were prepared by inoculating 5 ml SDB by a loopful of a colony from the freshly prepared yeast culture plates, followed by incubation at 32.5°C. Dextrose and sucrose were used for the spot dilution test in order to find out the osmotic tolerance. For spot dilution tests, 1ml of the culture suspension at 60 min interval was removed and serially diluted in 9 ml dextrose and sucrose broth up to 10^{-4}. From each dilution 1 ml was spotted onto SDA plates following incubation at 32.5°C for 24 hours. To observe the osmotic effect on cell growth, different concentrations of dextrose including 0.04 g/l (1X), 0.12 g/l (3X), 0.2 g/l (5X), 0.28 g/l (7X), 0.36 g/l (9X) and sucrose, i.e., 0.02 g/l (1X), 0.06 g/l (3X), 0.1 g/l (5X), 0.14 g/l (7X), and 0.18 g/l (9X) were used. The optical density at 600 nm (OD_{600}) and the capability to form the colony-forming units (CFUs) were monitored at the specific time intervals. For morphological observation, an aliquot of 5µl from each of the culture suspension was removed at 60 mins intervals. All experiments were conducted in triplicates (Munna, et al., 2015).

2.2.7 Gastric acid tolerance

The Organism was grown in SDB for 48 hour. Freshly prepared culture of *Candida shehatae var shehatae* of 1ml added in different pH of gastric juice. Stimulated gastric juices were prepared fresh daily by suspending [As per USP, 3.2gm/L, NaCl 2gm/L, HCl (for adjusting the pH value)]. Freshly prepared stimulated gastric juices were prepared at pH of values 2, 2.5, 3, 3.5, 4, and 4.5. In order to find out the probiotic activity of *Candida shehatae var shehatae* pH values were selected as per the expected and related pH of gut microbiota. The culture was subjected to its acid tolerance by subjecting it to artificial gastric juice. Suspension of 1 ml culture was transferred in a 5 ml sterile tube and was mixed in 0.5 ml of 0.5% sterile NaCl solution and 2 ml stimulated gastric juice of pH 2, 2.5, 3, 3.5, 4 and 4.5 respectively and was incubated at 32.5°C for 24 and 48 hours. (Slightly modified) (Hassanzadazar, et al., 2012). The optical density at 600 nm was monitored at different time intervals. The viability of strains was analyzed by determination of CFU/ml after different periods of incubation (0 min, 60 min, 120 min, 180 min) in the stimulated gastric juice again at 32.5°C for 24 hours.

2.2.8 Bile salt tolerance

Yeast cultures were grown on SDB, after incubation at 32.5°C for 48 hours. The colonies were suspended in a 0.5% NaCl solution. Suspension of 1 ml culture was transferred in a 5 ml sterile tube and mixed with 2 ml bile salt solution in different concentrations (0.1%, 0.5%, and 1%) and incubated at 32.5°C for 24 and 48 hours. The optical density at 600 nm was measured at different time intervals. The viability of strains was analyzed by determination of CFU/ml after different periods of incubation (0 min, 60 min, 120 min, and 180 min) in bile salt concentrations at 32.5°C. (Slightly modified) (Hassanzadazar, et al., 2012).

2.3 Statistical Analysis

All experiments were carried out in triplicate. Statistical analysis regarding yeast growth was performed by determining the P value. Standard deviations were also measured with the aid of statistical hypothesis testing for the indicated number of independently performed experiments.

Chapter 3

Results

3.1 The BIOLOG YT microplate test:

BIOLOG microbial identification systems software is used to identify the yeast from its phenotypic pattern in the YT Microplate. The isolate was identified by this method.

3.1.2 Identification of yeast by BIOLOG system

Result had been interpreted by BIOLOG. Microplate containing yeast sample observed by using MicroLog 3 Software which was given in the instruction guide. The color density or turbidity increase in each well is referenced against the negative control wells, A-1 and D-1. Wells that has a noticeable increase in absorbance at 590 nm were scored as positive. All wells optically resembling the negative control wells are scored as "negative" (-) and wells with an extremely slight increase in absorbance at 590 nm are scored as "borderline" (\). After 24 hours of incubation, the similarity sample gave an acceptable identification. After 72 hours of incubation, the similarity index was 0.625 which was considered to be acceptable. (Figure 3.3)

Isolate that was identified by BIOLOG system is explained in the following table.

Table 3.1: Isolates identified by BIOLOG™ system, collected from cheddar cheese sample from the local market.

Sample	Isolates ID	PROB (probability)	SIM (similarity index)	DIST (distance)	Organism type	Species
Cheddar cheese	2	0.686	0.625	0.343	Yt	*Candia shehatae var shehatae*

3.2 Suitable media for *Candida shehatae var shehatae*

Rose Bengal agar

Rose Bengal Agar is a selective medium to detect and enumerate yeasts and molds in food samples. A very few amounts of semi-white yeast colonies were observed on this medium. Colony shape and surface appearance were used for the confirmation.

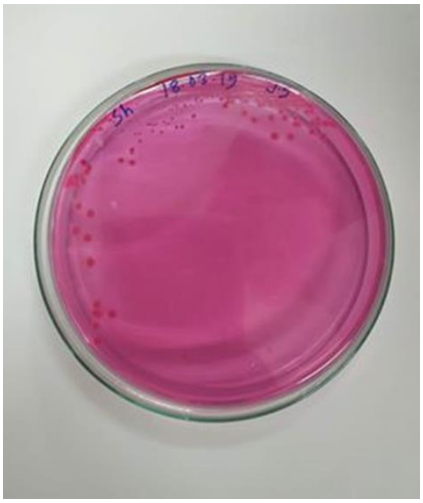


Figure 3.1: Growth on Rose bengal agar

Growth on YMA (Yeast Mannitol agar)

Little Amounts of colonies were found in this growth medium. Therefore this medium is not very suitable for the growth of *Candida shehatae var shehatae.*

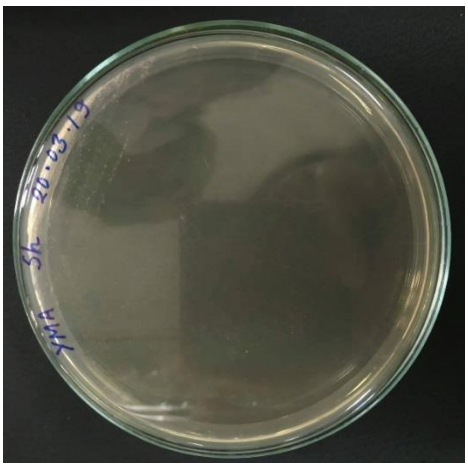


Figure 3.2: Growth on YMA

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	MRS 2
Sample Source	
Operator	
Microplate Lot No	3005121
IF Lot No	
Comments	
Date & Time of Read	Dec 10 2018 4:33 PM
Biolog ID DB	Yt60172.eid

Result	Species ID: Candida shehatae var shehatae
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.686	0.625	1.329	Yt	Candida shehatae var shehatae
2	0.249	0.221	1.672	Yt	Clavispora lusitaniae
3	0.023	0.020	2.476	Yt	Candida peltata
4	0.011	0.009	2.717	Yt	Pichia holstii

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	9	-2	-2	{ 43	{ 38	4	{ 27	17	8	{ 86	{ 56
B	< 383	< 92	< 169	< 404	< 219	-17	< 161	-25	-10	< 150	< 372	< 119
C	{ 77	< 178	< 252	8	{ 28	< 204	{ 55	{ 36	13 +	< 102	{ 30	-8
D	0	21	15	12	{ 33	-2	-12	7	{ 58	3	{ 27	{ 32
E	{ 64	{ 82	{ 47	{ 75	{ 64	-23	{ 49	-19	-18	{ 47	< 98	{ 44
F	{ 27	{ 31	{ 53	{ 84	-16	-18	-11	-24	{ 83	4	{ 34	16
G	7	{ 41	{ 50	-20	-5	{ 39	-16	-3	-13	-18	-18	2
H	< 139	{ 47	5	6	11	10	5	6	{ 73	2	6	-12

Report Date Dec 10 2018 4:33 PM

Figure 3.3: Identification of the organism in BIOLOG

Growth on YPD agar and YPD broth

YEPD or yeast extract peptone dextrose, also often abbreviated as YPD, is a complete medium for yeast growth. Semi-white yeast colonies were observed on YPD agar medium. Turbidity in YPD broth also had been observed.

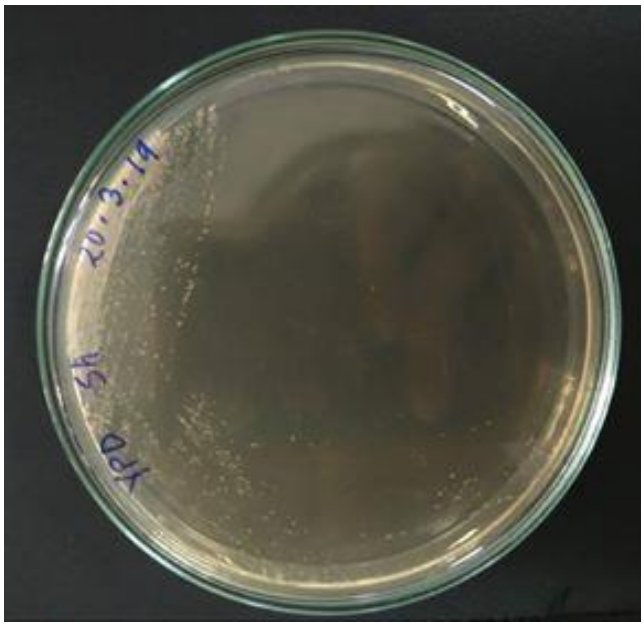
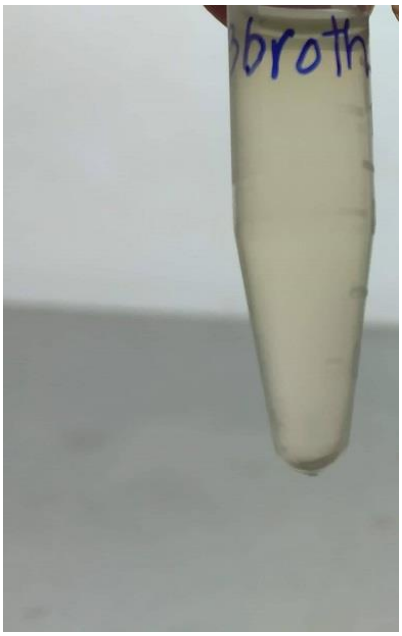


Figure 3.4: a) Growth on YPD Agar



b) Growth on YPD broth

Growth on SDA (Sabouraud Dextrose Agar) and SDB (Sabouraud Dextrose broth)

Smooth semi-white like yeast colonies with butter like consistency were observed on SDA plate. Colony shape and surface appearance were used for the confirmation. Pellets and turbidity also observed in SDB.

Figure 3.5: Growth on SDA medium

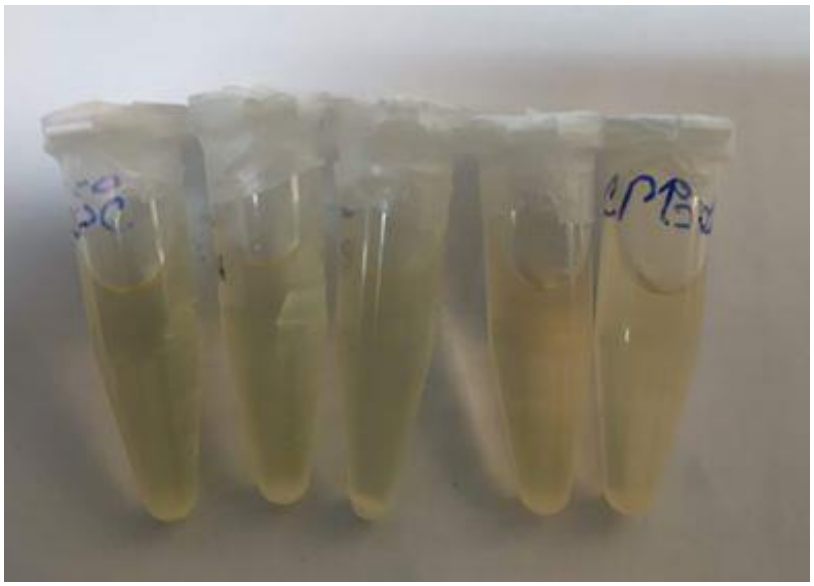


Figure 3.6: Growth on SDB

3.3 Microscopic observation

Compound microscope was used to observe the cell morphology of yeast (*Candida shehatae var shehatae)* isolated from cheddar cheese. Vegetative reproduction via budding was detected. Yeast isolates from the cheese sample was oval in shape and sometimes it showed rod like structure at the time of reproduction.

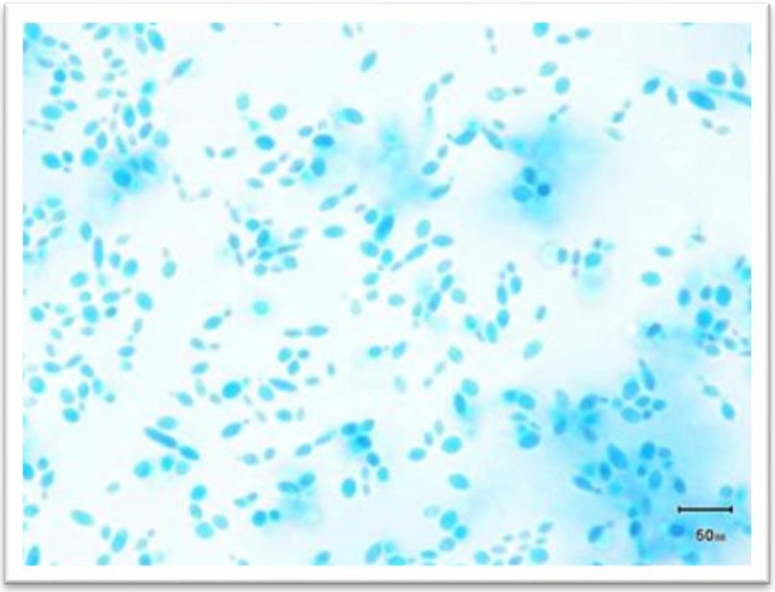


Figure 3.7: The cell morphology under compound microscope (100X)

3.5 Optimum growth temperature

Candida shehatae var shehatae this organism showed its rapid growth at 32.5°C. So this temperature can be called as an optimum growth temperature for this organism.

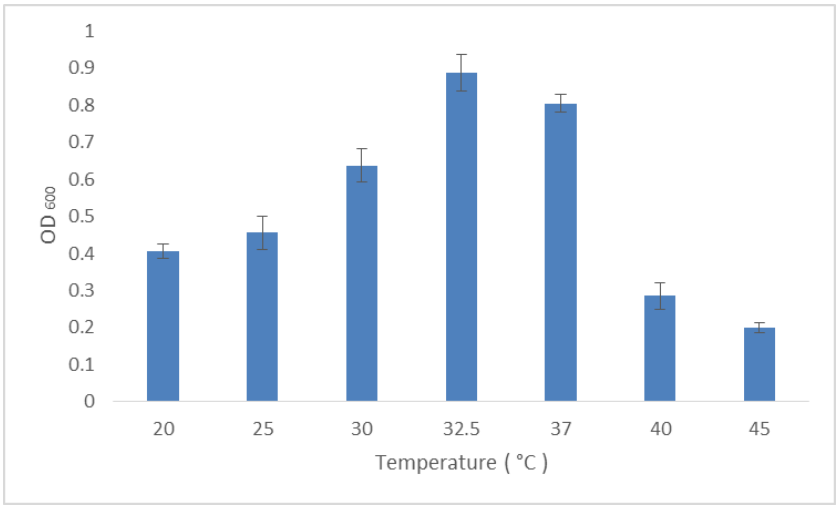


Figure 3.8: Optical density of the yeast at different temperature

3.6 Salinity and pH

Salt tolerant was observed in terms of this yeast. Moderate to high concentrations of salt the yeast can withstand and it showed high peak at 6%.

Candida shehatae var shehatae organism was observed in acidic solution. It showed high peak at pH 5.5 and absorbance went down after pH 7.

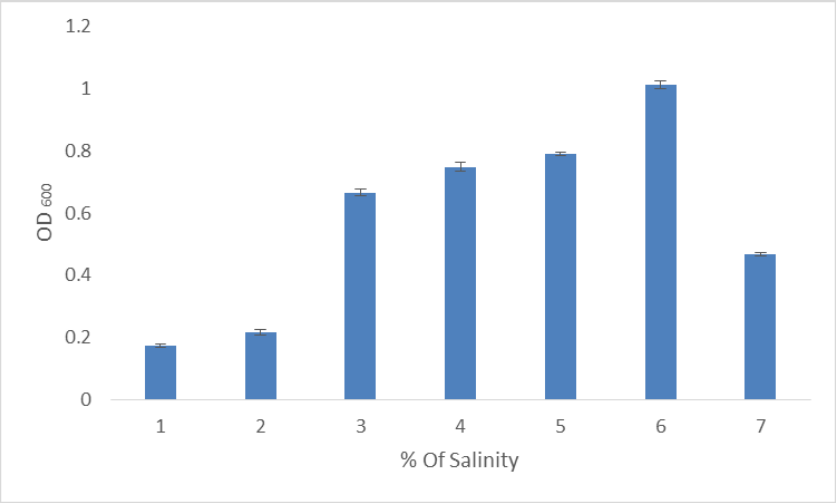


Figure 3.9: Salinity tolerance of the yeast

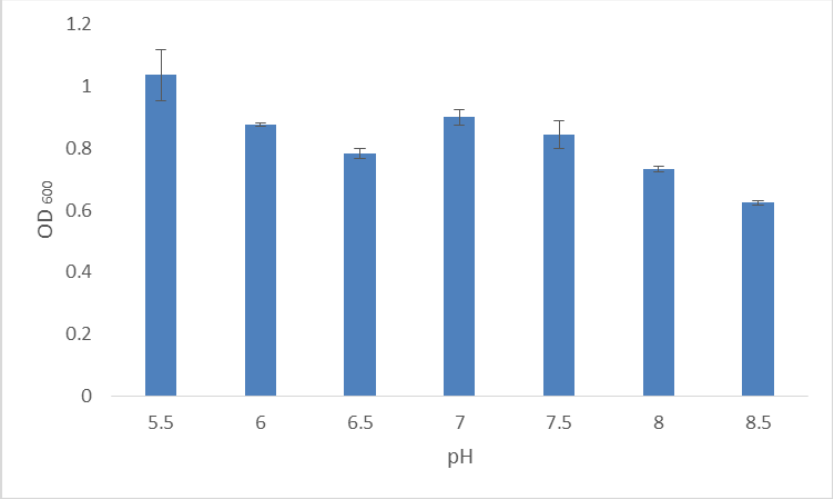


Figure 3.10: Absorbance of the yeast at different pH

3.7 Heat stress tolerance

The optimal growth temperature for *Candida shehatae var shehatae* was assessed through the measurement of OD_{600} and by counting the CFUs up to 300 min. After 60 mins of incubation at 32.5 °C the cell number was found to increase rapidly whereas at 30 °C such a tendency was a bit slower. However, compared with the growth stage at 32.5 and 30 °C, a relatively long lag

phase (~120 min) was observed when cells were grown at 37 and 40 °C, possibly due to the requirement of long time to cope with temperatures higher than the optimal growth condition. Besides, under the light microscope, budding yeasts were observed after 120 min at 30 and 32.5°C, whereas after 180 min such budding was observed at 37 °C. At 40 °C, the budding events were noticed after 240 min (Fig. 3.13). CFU cell counts after 48 hours of incubation showed highest growth at 32.5°C.The optimal growth temperature for *Candida shehatae var shehatae* was thus noted to be 32.5 °C.

However, the budding yeasts were found to become dormant when cells were grown at 45 °C. Also at 45°C growth on the plates showed very few colonies and absorbance was relatively low compared to other temperatures. Hence the critical growth temperature of this strain was recorded at 45 °C.

Table 3.2: CFU cell counts after 48 hours of incubation at different temperature

Temperature	CFU/ml	Log cell counts
30°C	1.33×10^{6}	6.12
32.5°C	**1.67×10^{6}**	**6.22**
37°C	9.8×10^{5}	5.99
40°C	6.7×10^{5}	5.82
45°C	7.0×10^{4}	4.84

Table 3.3: CFU cell counts after 72 hours of incubation at different temperature

Temperature	CFU/ml	Log cell counts
30°C	**2.53×10^{5}**	**5.40**
32.5°C	2.07×10^{5}	5.31
37°C	1.8×10^{5}	5.25
40°C	1.7×10^{5}	5.23
45°C	6.0×10^{4}	4.77

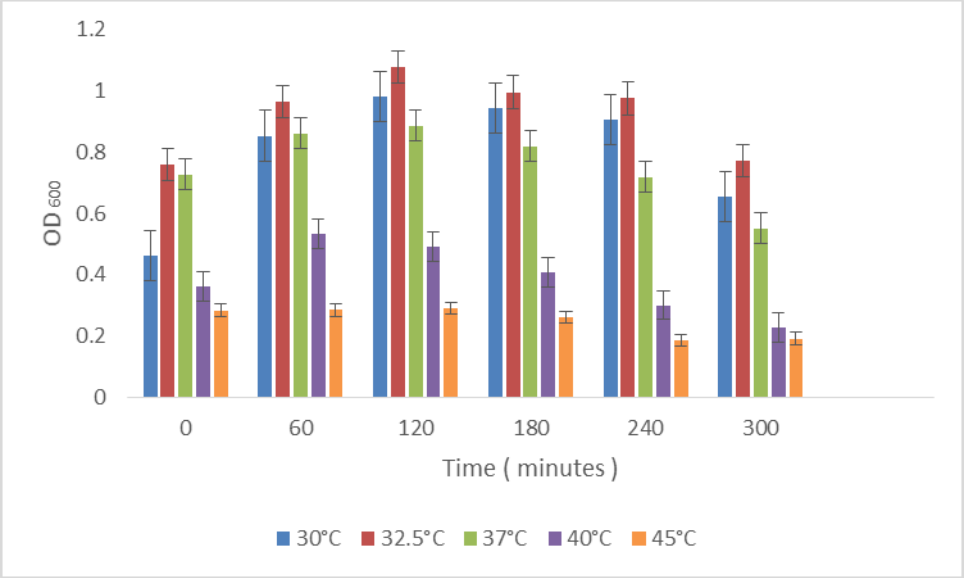


Figure 3.11: Absorbance at different time (minuets) after 48 hours of incubation

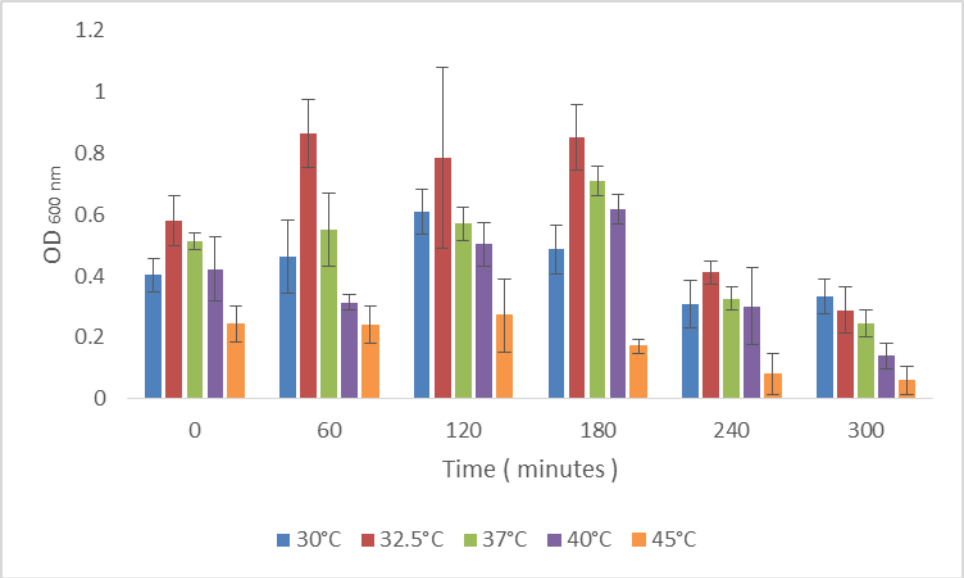


Figure 3.12: Absorbance at different time (minuets) after 72 hours of incubation

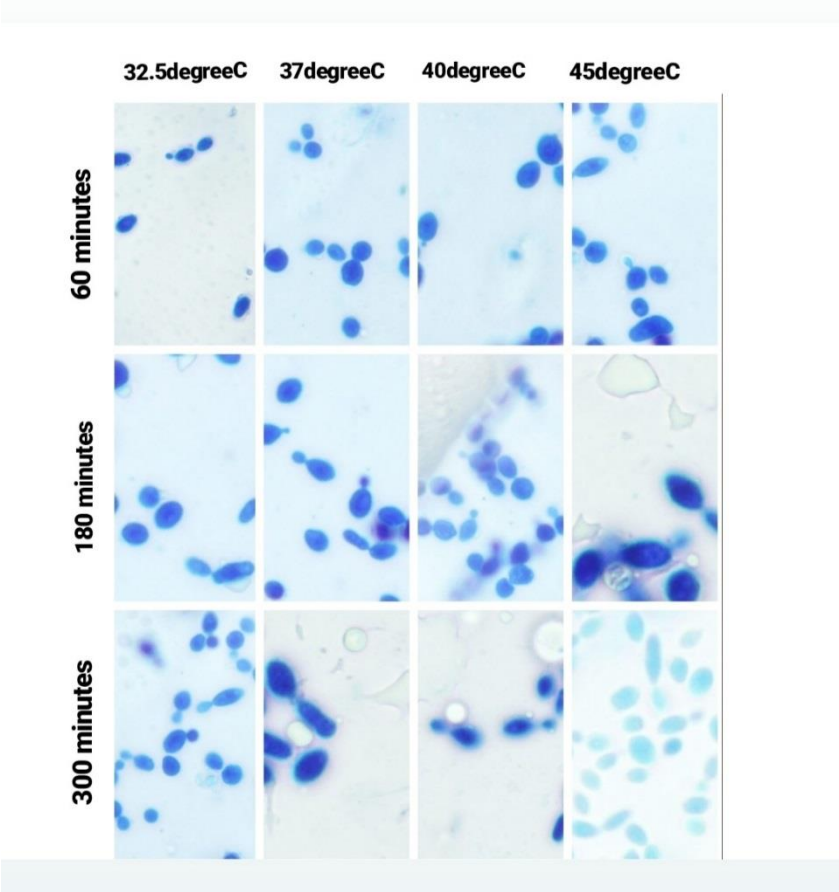


Figure 3.13: Morphological study of *Candida shehatae var shehatae* cells at different time intervals after incubation for 48 hours at optimum 32.5°C and high 45°C. Aliquots were removed at 60, 180 and 300 min for the assay. Active budding yeast was observed under light microscope at 32.5 °C up to 300 min of incubation. Besides, stressed cells (cells without the cytoplasmic contents) were observed at 45°C after 300 min of incubation

3.8 Osmotic stress tolerance

Different levels of osmotic pressure were simulated onward in order to achieve the complete stress response consequences of *Candida shehatae var shehatae* upon critical temperature. A relatively long lag phase was observed in dextrose concentration from 120 min-240 min at optical density at 600 nm. After 240 min, phase was observed to be going down. On the other hand, in sucrose concentration. Approximately 4 log CFU/ml was found to be abolished in 7X and 9X dextrose concentrations as well as in sucrose concentration.

According to earlier investigation also showed that yeast cells may exhibit an immediate growth arrest when exposed to an increase in external osmolality. Interestingly in the morphological study, the budding cells found to become quiescent at the optimal temperature with 3X dextrose concentration. Cells also found to be thickened at 5X concentration and become dormant at 9x after 300 min of incubation.

Table 3.4: Cell counts at different dextrose concentrations and at different time intervals after incubation for 48 hours at 32.5°C

Dextrose (Concentration)	0 min (CFU/ml)	60 min (CFU/ml)	120 min (CFU/ml)	180 min (CFU/ml)	240 min (CFU/ml)	300 min (CFU/ml)
1x	3.9×10^5	7.7×10^5	**4.6×10^6**	3.36×10^6	2.96×10^6	2.54×10^5
3x	4.1×10^5	7.1×10^5	4.2×10^6	2.32×10^6	2.76×10^6	2.31×10^5
5x	4.5×10^5	6.5×10^5	1.28×10^6	3.72×10^6	2.60×10^6	1.23×10^5
7x	3.0×10^5	4.3×10^5	1.31×10^6	3.6×10^6	3.16×10^6	1.6×10^5
9x	2.7×10^5	2.1×10^5	1.25×10^6	2.3×10^6	2.84×10^6	1×10^5

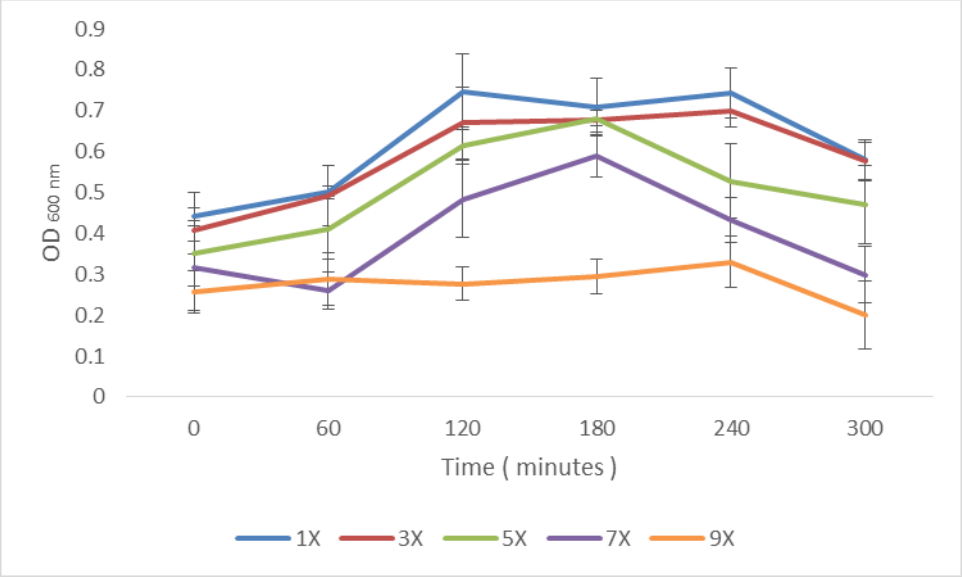


Figure 3.14: Growth relation of *Candida shehatae var shehatae* at 32.5°C with different dextrose concentrations at different time intervals after 48 hours of incubation

Table 3.5: Cell counts at different sucrose concentrations and at different time intervals after incubation for 48 hours at 32.5°C

Sucrose (Concentration)	0 min (CFU/ml)	60 min (CFU/ml)	120 min (CFU/ml)	180 min (CFU/ml)	240 min (CFU/ml)	300 min (CFU/ml)
1x	3.1×10^5	6.4×10^5	1.76×10^6	**3.96×10^6**	1.67×10^6	1.44×10^5
3x	3.9×10^5	6.2×10^5	1.78×10^6	4.36×10^6	1.20×10^6	2.61×10^5
5x	4.1×10^5	7.5×10^5	1.20×10^6	3.92×10^6	1.80×10^6	1.83×10^5
7x	3.6×10^5	5.8×10^5	1.36×10^6	3.55×10^6	3.56×10^6	1.92×10^5
9x	2.3×10^5	2.9×10^5	2.00×10^6	2.1×10^6	1.84×10^6	1.3×10^5

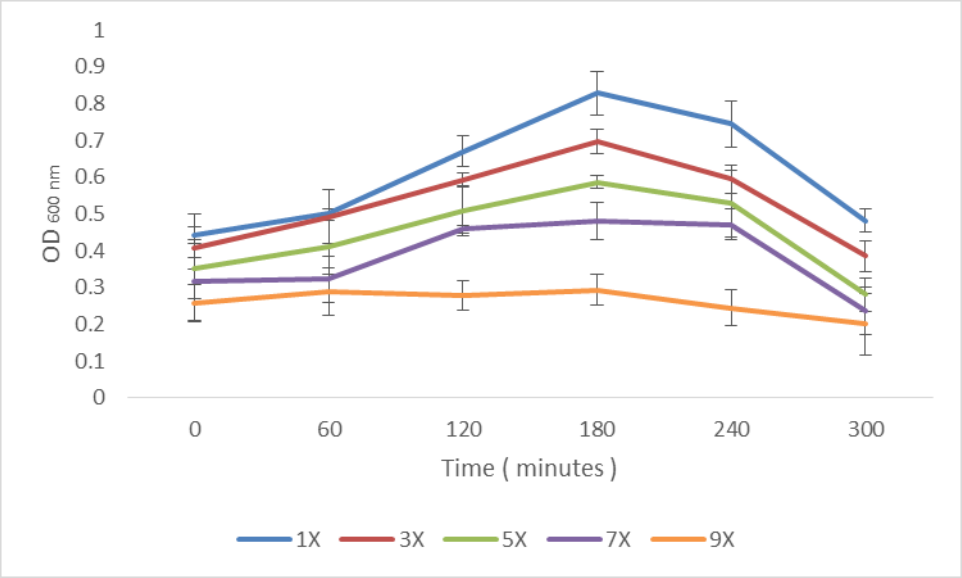


Figure 3.15: Growth relation of *Candida shehatae var shehatae* at 32.5°C with different sucrose concentrations at different time intervals after 48 hours of incubation

Morphological pattern:

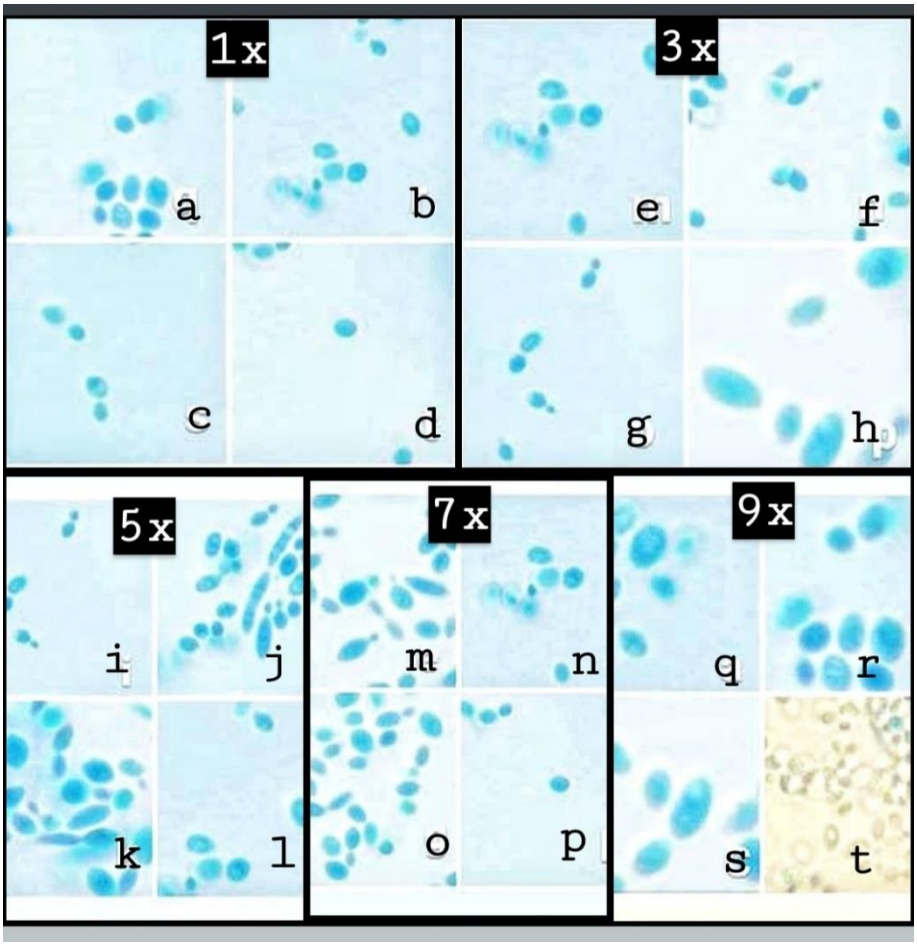


Figure 3.16: Morphological pattern at different dextrose concentrations. 1X,3X,5X,7X,9X-a, e, I, m, q (60 min), b, f, j, n, r (180 min), c, g, k, o, s (240 min), d, h, I, p, t (300 min)

3.9 Gastric juice tolerance

The promising activities of yeasts as well as their ability to survive during the passage through the human gastro-intestinal tract, tolerating exposures to low pH and to bile salts, have drawn attention to their possible use as probiotics (Lourens-Hattingh & Viljoen, 2001), even if their use for humans is currently restricted.

Candida shehatae var shehatae showed its activity towards the estimated low pH in artificial gastric juice. It was observed that the organism showed high growth at pH 3 and 3.5 in 600 nm absorbance compared to other pH levels and also CFU noted up to log 4 phase at higher growth.

After 24 hours at different time intervals the growth was observed at its high peak at 600 nm absorbance from 60 mins to 120 mins.

As *Candida shehatae var shehatae* showed its optimum growth after 48 hours of incubation so after 48 hours of incubation in artificial gastric juice it showed the highest peak at pH 3.5.

Table 3.6: CFU cell counts of *Candida shehatae var shehatae* in artificial gastric juice at different time intervals after incubation for 24hours at 32.5°C

pH	0 min (CFU/ml)	60 min (CFU/ml)	120 min (CFU/ml)	180 min (CFU/ml)
3	6.3×10^{5}	2.4×10^{7}	$\mathbf{1.96\times10^{7}}$	3.3×10^{5}
3.5	9.0×10^{5}	7.0×10^{6}	8.4×10^{6}	5.0×10^{5}
4	1.00×10^{5}	1.01×10^{5}	1.06×10^{6}	1.8×10^{5}

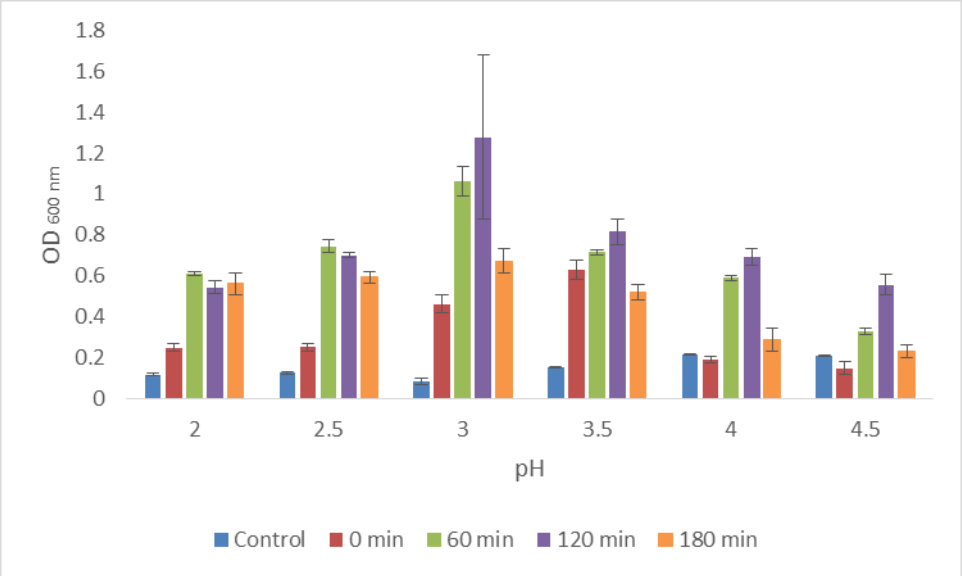


Figure 3.17: Gastric juice tolerance of *Candida shehatae var shehatae* at different time intervals after 24hours of incubation at 32.5°C

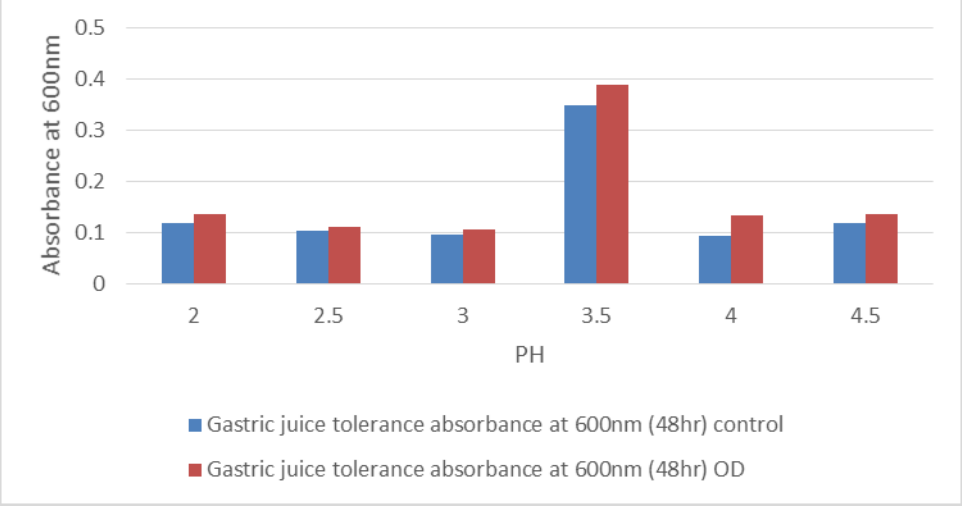


Figure 3.18: Gastric juice tolerance of *Candida shehatae var shehatae* after 48 hours of incubation at 32.5°C

3.10 Bile salt tolerance

The isolated yeast was observed at three different bile salt concentration 0.1%, 0.5%, 1%. After 24 hours of incubation in different bile salt concentration *Candida shehatae var shehatae* showed its highest peak at 60 min 0.5%. It had also shown good growth at 1% concentration.

Table 3.7: CFU cell counts of *Candida shehatae var shehatae* after 24 hours of incubation at different bile salt concentrations at different time intervals

Concentration of bile salt	0 min (CFU/ml)	60 min (CFU/ml)	120 min (CFU/ml)	180 min (CFU/ml)
0.1%	3.12×10^{6}	2.89×10^{7}	2.8×10^{5}	1.3×10^{5}
0.5%	2.56×10^{6}	1.47×10^{7}	2.1×10^{5}	2.7×10^{5}
1%	6.0×10^{5}	**7.7×10^{6}**	4.2×10^{5}	3.5×10^{5}

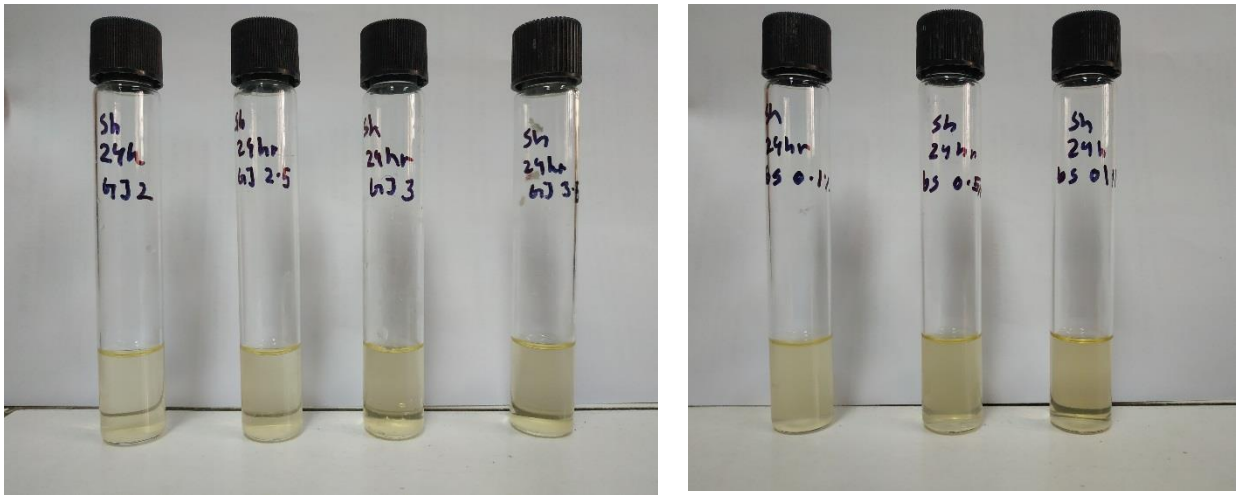


Figure 3.19: Gastric juice and Bile salt at different concentrations

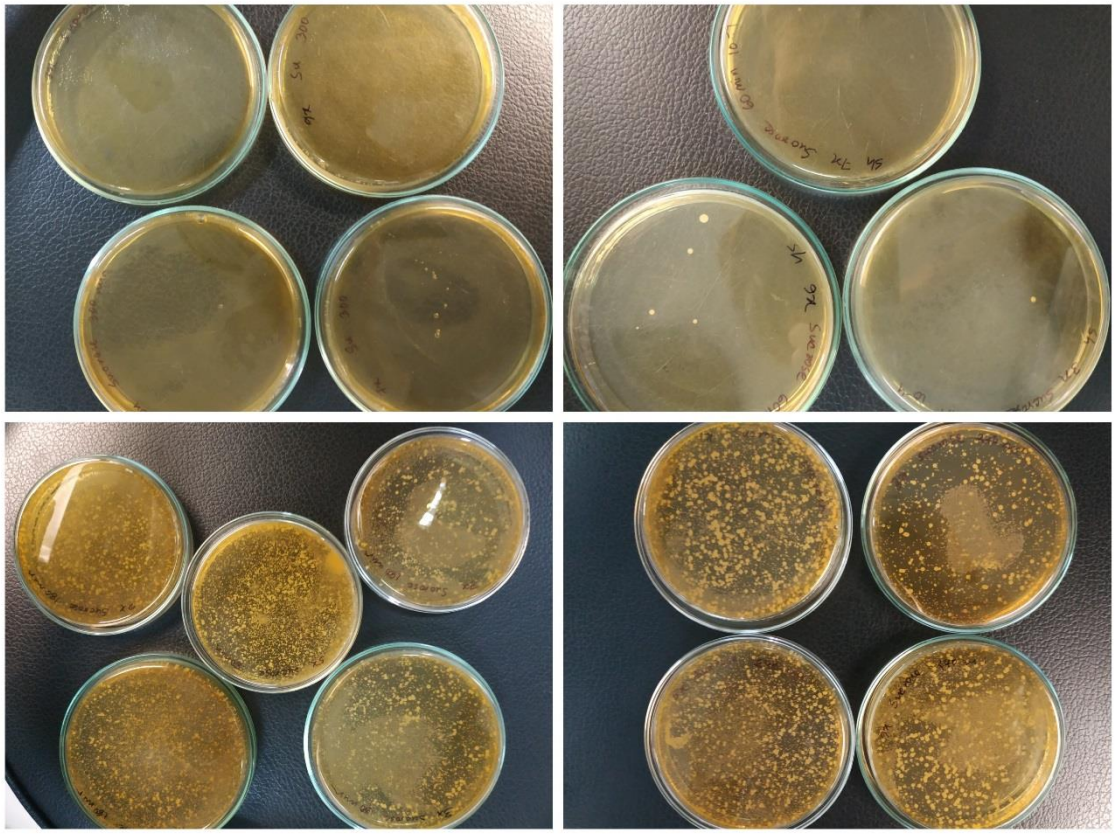


Figure 3.20: The growth of *Candida.sh* at different sucrose concentrations on SDA medium

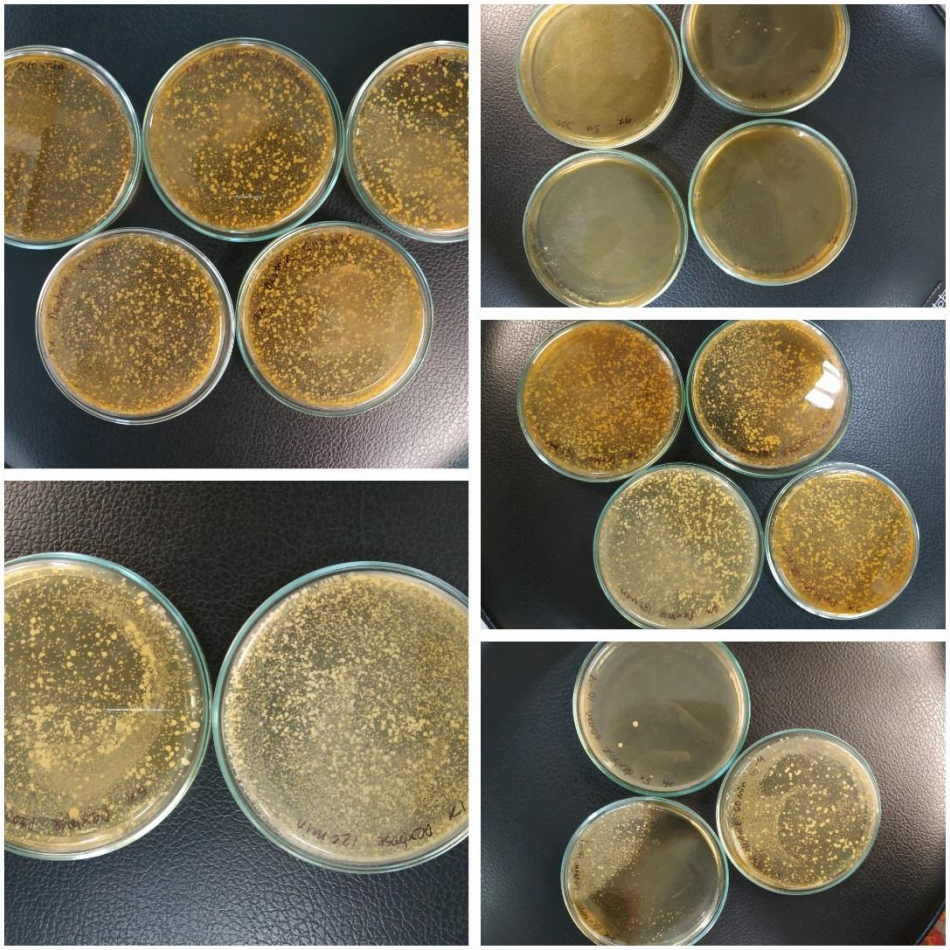


Figure 3.21: The growth of *Candida.sh* at different dextrose concentrations on SDA medium

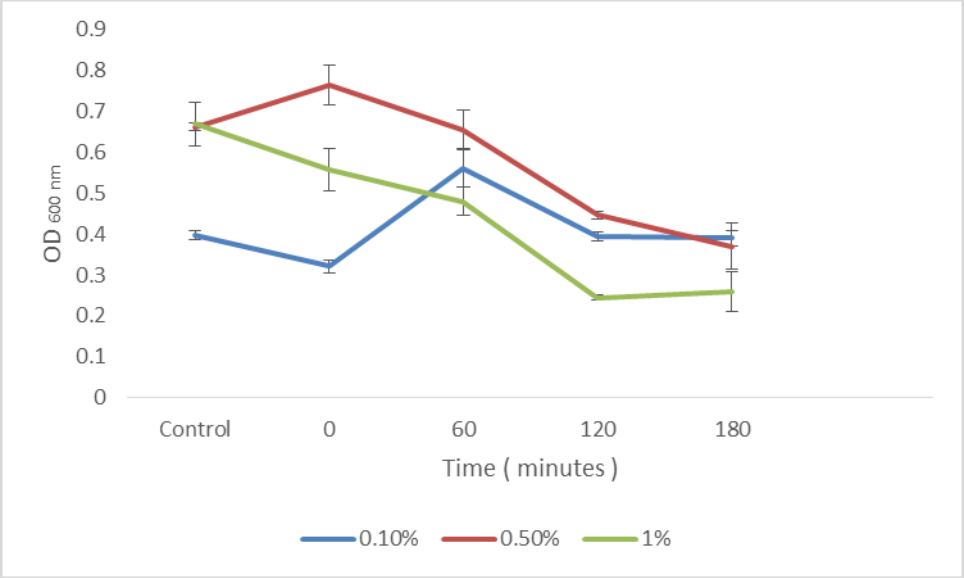


Figure 3.22: Bile salt tolerance level of *Candida shehatae var shehatae* at different time intervals after 24 hours of incubation at 32.5°C

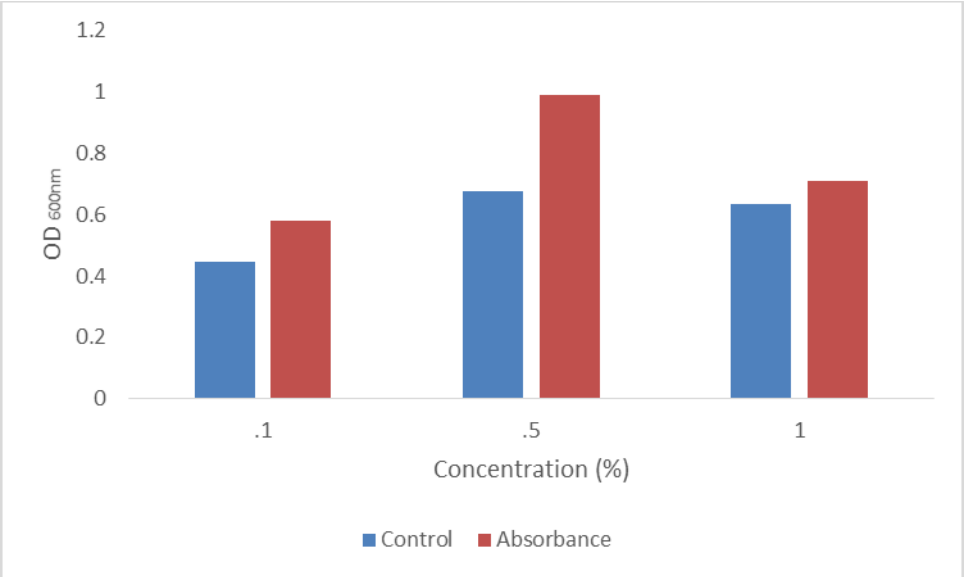


Figure 3.23: Bile salt tolerance level of *Candida shehatae var shehatae* at different time intervals after 48 hours of incubation at 32.5°C

Chapter 4

Discussion

4.1 Discussion

Though local isolates are usually more adapted but now a days several industrial yeasts are also available. In this study, yeasts were isolated from cheddar cheese from local resources.

This study was conducted in order to find out the optimum growth temperature, media, pH level, and how this organism tolerates high temperature to find out the death phase and how the organism functions at different concentrations of osmotic pressure.

According to the study of (AL Zubaidy & Khidhr , 2014) the isolation method was done by using the Sabouraud dextrose agar and the strain was isolated from the fruit. In this study, the strain was collected from a local cheddar cheese sample using the Rose Bengal agar. Another study by (Moeini, et al., 2004), yeast strains were isolated from dairy products.

Also in this study carried by (AL Zubaidy & Khidhr , 2014), identification of isolated yeast was done by biochemical tests such as Carbon source fermentation, Nitrogen source utilization, acid production from fermented sugars, ester production, urea hydrolysis were tested to isolate yeasts. Whereas in this study, the identification of isolated yeasts was done by BIOLOG identification software. Many studies have been conducted where the identification process was based on different chemical tests including the fermentation of sugars, liquid assimilation of carbon compounds or many more. Hence BIOLOG identification system is the easy and fast process where the software identified the organism based on its reaction and color change formation on the selected YTmicroplate.

There are many culture media for the yeast. The composition of the medium used for the cultivation of micro-organisms is directly reflected in their physiological phenotype and their fermentation performance. In this study the identified *Candida shehatae var shehatae* organism cultured in SDA medium which is Sabouraud dextrose agar and SDB (Sabouraud dextrose broth).

The optimum temperature for yeast growth is 32°C. Yeast cell death occurs above 38°C. The optimum temperature range for yeast fermentation is between 90˚F-95˚F (32˚C-35˚C). Many studies have conducted where the optimum growth for yeast was considered from 25-37°C. The optimum growth temperature for yeast strain was considered to be 25°C (Moeini, et al., 2004) *Saccharomyces cerevisiae* optimum growth temperature range was considered 32.5°C according to (Munna, et al., 2015). The optimum temperature of *Candida shehatae var*

shehatae showed 32.5°C in this study. This is the suitable growth temperature for this yeast strain.

In a study carried out by Munna *et al.,* in 2015, the yeast strain showed the optimum growth temperature at 32°C. They also observed cell growth at 37 and 40 °C, possibly due to the requirement of a long time to cope with temperatures higher than the optimal growth condition. Budding yeast also observed under the light microscope. They also reported that the ability of the yeast strain *Saccharomyces cerevisiae* grow at 40 °C while a sharp drop was observed in CFU and a relatively lower reduction in the cell turbidity was noticed at 44 and 45 °C. Interestingly, the budding yeasts were found to become dormant when cells were grown at 45 °C. In consistent with the results from the growth assessment, all yeast cells were found to lose the culture ability completely at 46 °C as observed through the spot dilution tests. Hence the critical growth temperature of this strain was recorded at 45 °C. Another study carried out by (Moraitis & Curran, 2004) they reported how reactive oxygen species may influence heat shock response and stress tolerance in the yeast *S. cerevisiae*. In the current study, *Candida shehatae var shehatae* strain showed the same result under heat stress. Based on previous study after observing the growth level and cell turbidity at 44 and 45 °C in this study, budding yeasts also found to become dormant when cells were grown at 45 °C. Based on the study the critical growth temperature of *Candida shehatae var shehatae* was recorded at 45°C.

Further studies have shown that, in order to achieve the complete stress response consequences of *S. cerevisiae* upon critical temperature, different levels of osmotic pressure were simulated onward. Same study conducted in this project where the yeast strain was further examined in order to find out the osmotic stress level. At 32.5°C a lag phase was observed in extended dextrose concentration. Approximately 4 log CFU/ml was found to be abolished in 7X and 9X dextrose concentrations. When the medium was supplemented with extremely high (9X) dextrose concentration at 45°C cells were found to lose their culturability completely. Budding cells were found to become quiescent at low concentrations and thickened at high concentrations. In this study, the current findings showed relatively long lag phase and the cells started to become thickened in 7X concentration and found to become dormant at 9X concentration. In cohesion with the current findings, previously, the cell volume of *S. cerevisiae* was also found to expand at 48 h of incubation periods upon osmotic stress. Hence based on this study the cell volume also found to expand after some time being of incubation. However, a huge number of cells losing the cytoplasmic contents were observed at 45 °C with extremely high (9X) dextrose concentration. Consistently, in the absence of osmotic

imbalances no stressed cells (cells without the cytoplasmic contents) were observed at 45 °C and complete growth suppression due to osmotic shock. Another study conducted by (Beney, et al., 2001) provided the evidence, which showed that increases in osmotic pressure resulted in corresponding decreases in yeast viability. The findings also showed changes to the shape and features of the yeast cells.

The concept of probiotics (which means, "for life") was introduced in the early 20th century by Elie Metschnikoff, it however, gained momentum only recently with considerable and significant advances in the functional and health food market across the world. Certain criteria should be met before a microorganism is selected as a probiotic. There are some criteria to fill up as probiotic those are it should be a strain that is capable of exerting a beneficial effect on the host consuming it. The strain should be non-pathogenic and non-toxic, it shouldn't be harmful to the host itself. Also it should be able to withstand in foodstuff at high cell counts and be viable throughout the shelf life of the product. There are also some characteristics, the strain should be able to adhere to the intestinal cell lining and be able to survive in gut conditions and colonize there and it should produce antimicrobial substances towards pathogens. Those are the criteria for good probiotic (Afrc, 1989). In a study carried out by (AL Zubaidy & Khidhr , 2014) to find out the probiotic activity of *Saccharomyces cerevisiae var. boulardii* they conducted some in vitro process. Whereas gastric juice tolerance level was noted and showed that their isolated yeast strains can resist acidity, and the strains have different ability to resist low pH. Some yeasts showed the highest percentage of survival also tolerated acidity against some other yeast strains. The pH of excreted HCl in the stomach is 0.9, but the presence of food raises the pH to 3.0 (Erkkilä & Petäjä, 2000). In the current study, the organism *Candida shehatae var shehatae* showed its activity towards the estimated low pH in artificial gastric juice. The organism showed its highest absorbance at pH 3 and 3.5 which means it has high efficiency to survive during the passage through the human gastro-intestinal tract, tolerating exposures to low pH.

Microorganisms that survive in the acidic condition of the stomach also have to survive in intestinal secretion and the bile salts in the duodenum (Erkkilä & Petäjä, 2000). Bile is a yellow-green aqueous solution whose major constituent includes bile acids, cholesterol phospholipids and pigment biliverdin. Bile resistance is an important criterion in the selection of culture as a dietary adjunct (Gilliland & Walker, 1990) because it could allow the growth of the ingested probiotic microorganism in the intestinal tract and the bile salts in the duodenum (Erkkilä & Petäjä, 2000).

In this study, the yeast strain (*Candida shehatae var shehatae*) showed its tolerance level to bile salt up to 1%. Hence based on the findings of this study it can be said that *Candida shehatae var shehatae* showed a positive result in gastric juice and bile salt tolerant test where it showed tolerance level in both these tests which are in vitro test for finding out the probiotic activity.

4.2 Conclusion

In conclusion it can be said that the yeast strain has the capability to tolerate the stress of osmotic pressure and can survive at critical growth temperature. Also this yeast strain has good probiotic characteristics in terms of in vitro analysis. Although further studies need to be carried out to find out the antimicrobial activity. Further studies should strongly focus on more in vitro analysis of probiotic activity and also in vivo analysis. Further screening of probiotics is always necessary to discover strains with very good probiotic characteristics.

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Appendix

Laboratory Instruments used throughout the study

Incubator	Memmert
Freeze	Siemens
Micro centrifuge	Mikro 120
Microscope	Olympus BX41
Weighting balance	A &D company LTD.
pH meter	Hanna Instruments
Laminar flow cabinets	ESCO
Micropipette	Eppendorf
Laboratory Glass bottles	Schott duran
Conical Flasks	Pyrex
Petri dishes	Sterilin
Autoclave	Systec
Spectrophotometer	PG Instruments(T-60 UV-Visible)