EXPLORATION OF YEAST *Candida zeylonoides* WITH SPECIAL REFERENCES TO PROBIOTIC ACTIVITY ISOLATED FROM MOZZARELLA CHEESE

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A Thesis Submitted to the Department of Mathematics & Natural Sciences in Partial Fulfillment of the Requirements for the Degree of Bachelors of Science in Biotechnology

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Declaration

It is hereby declared that,

1. The thesis submitted is my original work while completing 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 which has been accepted, or submitted, for any other degree or diploma at a university or other institution.

4. I have acknowledged all main sources of help.

_____

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Approval

The thesis titled "EXPLORATION OF YEAST *Candida zeylonoides* WITH SPECIAL REFERENCES TO PROBIOTIC ACTIVITY ISOLATED FROM MOZZARELLA CHEESE" submitted by Ritwika Shamshad of Fall, 2020 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology on

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Ethics Statement

The data presented in this thesis are acquired from conducted experiments. Information that are taken from other sources are appropriately cited through accurate referencing.

Abstract

Yeast strains have significant characteristics based on their nature and abilities to produce food products, medicines, protein supplements, etc. *Candida zeyalanoides* is the yeast strain that had been speculated in this study. This strain was collected from Mozzarella cheese and has an idiosyncratic nature of having a pink pigment of it. This is least speculated strain, so to identify some of its certain essences to certain experiments; for instance, its caliber to endure heat stress, its efficiency to undergo such osmotic stress and the probiotic efficacy of it was done along with its isolation, characterization. In a state of enduring different parity of temperatures (30°C, 32.5°, 37°C, 40°C and 44°C) the responses were diverse. However, *C. zeyalanoides* showed optimum growth at 32.5°C and the growth was less after the temperature reached higher than 40°C. Different concentrations of dextrose and sucrose solution were used to ascertain its ability to undergo osmotic stresses. In the dextrose solution, the growth was maximum in case of 7x concentration level and then the tolerance level had been dropped at 9x level. On the other hand, the growth level was fine at 5x level in case of sucrose and then gradually decreased. To ascertain the probiotic activity, gastric juice and bile salt tolerance test had been done. The test showed that the growth of *C. zeylanoides* or the value of absorbance decreased with the increase of salt concentration from 0.1% to 1%. In the case of the gastric juice test result, the cells can tolerate up to the pH level of 4.5 which means *C. zeylanoides* may have the probiotic activity strain in it.

Key words: *Candida zeyalanoides*, pink pigment, heat stress, osmotic stress, probiotic efficacy, pH level, optimum temperature, salinity.

Dedication

To everyone who prayed for my success.

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I would like to start by thanking the Almighty for keeping me in good health and giving me the strength to push through.

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Ritwika Shamshad

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List of Acronyms

SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
YPD Agar	Yeast Peptone Dextrose Agar
YPD Broth	Yeast Peptone Dextrose Broth
YMA	Yeast Malt Agar
TNTC	Too Numeric To Count

Chapter 1

Introduction

Chapter 1

Introduction and Literature Review

1.1 Introduction and Literature Review

Yeasts are the organisms that are single-celled microorganisms that have their nucleus enclosed within the membrane. The fungus is the classification kingdom for yeast. Although the yeast ancestor was multi-cellular, the yeasts are now becoming unicellular species by evolving. Yeasts constitute a very varied group of microorganisms, and even strains categorized as the same genus frequently show a high level of genetic divergence. The biodiversity of the yeasts is closely linked to their applicability. The biotechnological importance of yeast is almost beyond the account. People have used its enzymatic ability as bread or alcoholic beverages for centuries to produce fermented food. Admittedly the application of yeasts was originally instinctual, but with the development of science and technology, these microorganisms became the focus of rigorous scientific research. It should be acknowledged that yeast is an excellent scientific model due to its eukaryotic origin and expertise of yeast cell genetics, as well as the metabolic rate examined in specifics (Żymańczyk-Duda *et* al., 2017).

Yeast is widely dispersed with a wide range of habitats in nature. They are commonly found on leaves, flowers, and fruits of plants as well as in soils. Yeast can also be found on the skin surface and in the intestinal tracts of warm-blooded animals, where the animals can live symbiotically or as parasites. *Candida* is a yeast genus, and is the world's most common cause of fungal infections. Most organisms are harmless commensals or endosymbionts of hosts like humans; however, they may invade and cause disease, known as an opportunistic infection, when mucosal boundaries are damaged or the immune system weakened (Khoshravi *et al*., 2013). *Candida* is located along with the skin on most mucosal surfaces, mainly the gastrointestinal tract (Khoshravi *et al*., 2013).

In ever-changing environments, microorganisms adapted adaptive responses to withstand challenging threats. Here, this study has focused on those interactions in *C. zeylanoides* during adaptation to different stress challenges, focusing on heat stress responses and osmotic stress.

Yeast in several respects refers to the influence of deposition of the solute on the growth source. In reaction to osmotic pressures, yeast cells adjust their cell volume, decrease volume in reaction to hypertonic stress and raise volume in the presence of hypotonic stresses (Pratt *et al.*, 2003).

The species *C. zeylanoides* is a pink pigmented yeast cell that had been isolated from mozzarella cheese which is considered as the founding source of this organism for this thesis study. *C. zeylanoides* is a barely involved leaven in human pathology. This species is harmless commensals or endosymbionts. By testing the optimum growth of *C. zeylanoides*, it's bluntly clear that it can tolerate up to 37° C.

This thesis study is being performed to understand the species for closer details as there is very little work been done on this organism. This study has been done on *C. zeylanoides* to find out its changes, reactions heat stress, osmotic pressure, and the probiotic activity of it.

As yeasts are very fragile species to be handled, the tests were performed carefully so that other organisms could not contaminate the yeast. Yeasts are very prone to get contaminated by bacteria. So it was a tough task to tackle nurturing the fresh and pure colonies of *C. zeylanoides.* As with bacterial contamination, yeast-contaminated colonies become turbid, particularly if the contamination is at an advanced stage. There is very little shift in the pH of the yeast population until the exposure gets heavy, at which point the pH typically rises. Under microscopy, yeast occurs as ovoid or spherical individual particles, which may bud off smaller particles.

Typically, yeast colonies appear similar to bacterial colonies. Some species can evolve as white patches with a glossy surface, for example, *Candida*. The colonies of *C. zeylanoides* appeared as light off-white-colored small single colonies in the fresh culture. There is no specific smell of it which could be identified as its own smell. The colonies are of the normal size of yeast colonies and round shapes. The optimum growth hour is of 24 hours. Within 24 hours, the colonies appear in a fine way that could be cultured further for other purposes.

As much less work has been done on *C. zeylanoides*, the main goal of this thesis study is to find some information about this yeast strain to know some of their activity; namely, heat stress response and osmotic stress, finding the probiotic activity of this strain.

There are many microorganisms that are highly sensitive to heat. Too much heat will cause the death of the cells. Yeast adapts their ability to absorb changes in ambient temperature as necessary.

A correlation is known to exist in yeast and other organisms between the cellular resistance to stress and the life span (Swieciło *et al*., 2000). The purpose of this study was to examine whether or not stress treatment affects the yeast cells' generative life span. Cyclically applied both heat shock (up to 44° C) and osmotic stress (up to 9x dextrose strength as well as 9x sucrose strength) have been found to increase the mean and maximum life span of *C. zeylanoides.*

The goal of testing the heat stress level of this yeast strain is to determine the temperature level to which the strain would easily survive. This test is done in triplicates and the period of time to be observed was 24 hours, as the optimum growth time of it was 24 hours. This experiment has been done in the Sabouraud Dextrose Agar (SDA), the suitable media for its growth. The experiment is done at five different temperatures (30°C, 32.5°C, 37°C, 40°C and 44°C) to ascertain the suitable temperature along with the determination of the heat stress level of this species.

From these five temperature level, the strain survived very well up to 32.5°C, but then the growth started to become lower from the temperature from 37°C and at the ending temperature level there is a very little growth on the culture plate as the temperature is higher for the yeast strain to be survived in the temperature level of 40°C and 44°C. Mostly there is no colony been found on the culture plate at 44°C as the temperature is not suitable for the growth of *C. zeylaloides*. For this experiment, subculture is done in the Sabouraud Dextrose Broth (SDB) to use it for the dilution purposes and then for observing the colonies, plating is done in the Sabouraud Dextrose Agar (SDA). While inexorably age individuals of many types, a series of studies found that the aging process is modulated in response to a variety of mild stresses. Here, we were studying how heat stress facilitates yeast survival (Baldi *et al*., 2017).

The term probiotic (*pro* Lat. ‘for’ and *biotic&* Greek adjective from *bios* ‘life’) used by Werner Kollath in 1953 to denote all good organic and inorganic complexes, as opposed to harmful antibiotics. It is attributed to Lilly and Stillwel who in 1965 defined the probiotic as “a substance produced by one microorganism stimulating the growth of another microorganism”

(Łukaszewicz, 2012). Probiotics have been established in the prevention and treatment of various pathological circumstances as viable microorganisms that have a positive impact when ingested. Viable and biologically active microorganisms are typically required at the specified location of the host, so that it is necessary for the probiotic to transcend the host's natural defenses against ingested microorganisms (Czeruchka *et al*., 2007). Live micro-organisms that give the hosts a health benefit are probiotics. Bile resistance is an important criterion in the selection of culture as a dietary adjunct (Gilliland and Walker, 1990; Walker and Gilliland, 1993), because it could allow the growth of the ingested probiotic microorganism in the intestinal tract (Gilliland *et al*., 1984; Suskovic *et al*., 1997) and the bile salts in the duodenum (Erkkila and Petaja, 2000). It's synthesized in the pericentral hepatocytes of the liver, stored and concentrated in the gall bladder inter-digestively; the concentration used for the screening of a resistant probiotic strain is 0.3% w⁄ v (Gilliland *et al*., 1984). The gastrointestinal (GI) microflora ('microbiota') is an ecosystem that co-exists in equilibrium with its hosts. Clinical conditions may arise if this balance is disturbed (Czerucka *et al*., 2007). The low pH of the stomach, ranging from 2.5 to 3.5, is toxic to most microbes; it expands towards the distal part of the GI tract (Czeruchka *et al*., 2007). Sometimes it becomes difficult for microorganisms to survive in the gut after ingestion because then the food become exposed to the gastric juice. There have been many experiments performed to extract probiotic bacteria from different sources (Havenaar, 1992). The pH level there is high as the presence of the HCl acid concentration. The pH of excreted HCl in the stomach is 0.9, but the presence of food raises the pH to 3.0 (Erkkila and Petaja, 2000). Moreover, the presence of food or other food components could buffer the probiotics ingested, conferring some protective effect on the microbial cells in the stomach (Conway *et al*., 1987). The aim of the probiotic activity is to check the ability of *C. zeylanoides* to tolerate the lower pH of the GI tract. This test is being done by checking the Gastric juice and bile salt tolerance process to check whether this organism have a probiotic activity or not. The growth of *C. zeylanoides* showed a fine result on probiotic activity.

Many studies may have carried out over the years to isolate the various *Candida* species and study its various physiology and biochemical properties. But in the case of *C. zeylanoides* species, a few numbers of studies have been found related to its characteristics and utilities. Because of that their properties and different utilizations are not that much well-acquainted. This literature review will focus on certain characteristics of *C. zeylonoides* such as

its isolation, characterization, its caliber to endure heat stress, its efficiency to undergo such osmotic stress and the probiotic efficacy of it.

1.2 Isolation and Identification of *Candida zeynaloides*

The strain, *C. zeylanoides* was isolated from Mozzarella cheese. For enrichment of yeast in this sample, 5 gm of samples were homogenized in 0.1% peptone water and incubated at 30° C for 24 hours. Samples were diluted and poured onto the plate. The agar that was used here to identify the yeast sample was Sabouraud Dextrose Agar (SDA). After pouring samples onto the plate and the agar was added it was placed into the incubator at 30° C for 5 days. The colony-forming units (CFU) were determined after the incubation period. From the primary cultures, colonies with identical morphological appearance were selected for further purification. The purified yeast isolates were grown on the Sabouraud Dextrose Agar (SDA) medium and kept at 4° C until they were identified.

1.2.1 Competency to Endure Heat Stress

Many microorganisms are extremely heat sensitive. Too much heat can be causing cells to die. Like bacteria; yeast, mold also attempts to cope with the fluctuation of ambient temperature and thus the effectiveness of heat stress-sustaining yeast has been checked. But unlike bacteria yeasts in case of temperature variation are a little delicate to treat. Some yeast can grow at high temperatures but *C. zeylanoides* has a medium temperature scale to suitably grow colonies. It has a maximum or optimum temperature of 32.5°C. Although it can withstand temperatures of up to 40°C - 44°C in the growing period, rising slowly becomes less when the temperature range rises higher.

1.2.2 Efficiency to Undergo a Level of Osmotic Stress

Osmotic pressure is the force that develops separated by a semi-permeable membrane between two solutes of varying concentrations. When the yeast is exposed to an osmotic pressure is put

on it. Rather high osmotic pressures such as those found in high gravity can distort the metabolism of the yeast, or decrease the viability of the yeast. The degree of the osmotic pressure would depend on the distribution of the solutes around the cell. Increases in osmotic pressure have been shown to cause a concomitant reduction in yeast viability, development and fermentation efficiency.

1.2.3 Estimate the Presence of Probiotic Activity

Probiotics have been identified as viable microorganisms that have a positive impact when ingested, in the prevention and treatment of specific pathological situations. Viable and biologically active micro-organisms are typically required at the host's specified location, so that the probiotic can overcome the natural defenses of the host to ingested micro-organisms is important.

1.3 Objectives of the Study

- Assessing the deftness to endure heat stress of *C. zeylanoides*.
- Espial the tolerance level of the strain inwardly diversified concentration of dextrose and sucrose broth to review the cell structure.
- Determine the probiotic potential of the isolated *C. zeylanoides* species, whether it can abide the acidic situation in the GI tract.

Chapter 2

Materials and Methods

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Research Location

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

2.1.2 Isolates

In this study, isolates of *Candida zeylanoides* were used that obtained from the Industrial Microbiology lab and was isolated from the Mozzarella cheese and was identified with morphological and biochemical tests to be as species of *Candida*.

2.1.3 Observation of Colony Characteristics

The cell shape, size, color and other colony characteristics of the selected isolates were observed and tabulated.

2.2 Media and Reagents

2.2.1 Media

SDA and SDB were used in the further process of the experiments. While Rose Bengal Agar, YPD agar and YPD broth were previously used for the cultivation and measurement of *C. zeylanoides* production. But later on, SDA and SDB came out as the appropriate media for

growing the *C. zeylanoides*. On other media, this species had grown but the growth was less in contrast to the growth in SDA and SDB media. So SDA and SDB were selected as the preferred media for *C. zeylanoides* culture.

Sabouraud Dextrose Agar (SDA):

It is a selective medium and used mainly for dermatophyte isolation, certain fungi and yeasts, but also filamentous bacteria like the Nocardia may grow. The SDA medium is composed of the enzyme digest of casein and animal tissues that provide a nutritious source for the growth of fungi and yeast with amino acids and nitrogen substances.

Composition of Media:

Table 01: Composition of SDA Medium

Ingredients	**gm/L**
Mycological peptone (enzymatic digest of casein and animal tissues)	10 gm
Dextrose	40 gm
Agar	15 gm

Sabouraud Dextrose Broth (SDB):

Sabouraud Dextrose Broth (SDB) is as same as the Sabouraud Dextrose Agar (SDA). It is just the liquid solution that is being used to make broth culture and is also used for dilutions. Sabouraud dextrose is supplemented by peptone media with dextrose in order to support fungal growth. Peptone special provides the source of energy, nitrogen, vitamins, minerals, amino acids and factors of growth. Dextrose provides a source of microorganism growth energy. The low pH encourages fungal growth and impedes clinical specimens to contaminate bacteria.

Composition of Media:

Table 02: Composition of SDB of Medium

Ingredients	gm/L
Enzymatic digest of casein	5 gm
Enzymatic digits of animal tissue	5 gm
Dextrose (glucose)	40 gm
Peptone	10 gm

Peptone Buffered Water:

Peptone buffered water is used in a laboratory environment for the non-selective pre-enhanced diagnosis of *Salmonella spp*. Peptone buffered water is not for use in the treatment of sickness and other medical conditions. Peptone is a source of buffered peptone water nitrogen, carbon, vitamin and mineral. The osmotic balance of sodium chloride is preserved. The medium buffers phosphates.

Composition of Media:

Table 03: Composition of Peptone Buffered Water Medium

Ingredients	Gm/L
Peptone	10.0g
Sodium Chloride	5.0g
Disodium Phosphate	3.5g
Monopotassium Phosphate	1.5 g

Yeast Peptone Dextrose (YPD) Agar and Yeast Peptone Dextrose (YPD) Broth:

Yeast Peptone Dextrose (YPD) Agar and Yeast Peptone Dextrose (YPD) Broth are used in molecular microbiology procedures for the conservation and proliferation of yeasts. General yeast genetics methods specify the yeast extract-peptone-dextrose (YPD) medium used to grow *Saccharomyces cerevisiae* and other yeasts. As a cause of carbon, nitrogen, vitamins and minerals, YPD Agar and YPD Broth contain peptone. Yeast extract provides B-complex vitamins which stimulate the growth of bacteria. Dextrose is the source of the carbohydrates. YPD Agar includes the agar as the substantiating agent.

Composition of Media:

Table 04: Composition of YPD agar and YPD broth Medium

Difco™ YPD Agar:

Ingredients	**Gm/L**
Yeast Extract	10.0g
Peptone	20.0g
Dextrose	20.0 g
Agar	15.0 g

Difco™ YPD Broth:

YPD Broth consists of the same ingredients without the agar.

Yeast Malt Agar (YM Agar):

Yeast Malt Agar (YM Agar) is used for the isolation and cultivation of yeasts, moulds and other acid-uric microorganisms.

Composition of Media:

Table 05: Composition of Yeast Malt Agar (YM Agar) Medium

Ingredients	gm/L
Peptone	5.000
Yeast extract	3.000
Malt extract	3.000
Dextrose	10.000
Agar	20.000
Final pH (at 25°C)	6.2±0.2

Rose Bengal Agar:

Rose Bengal Agar Base is recommended for the selective isolation and enumeration of yeasts and moulds from environmental materials and food stuffs.

Composition of Media:

Table 06: Composition of Rose Bengal Agar Medium

Ingredients	gm/L
Papaic digest of soyabean meal	5.000g
Dextrose	10.000g
Monopotassium phosphate	1.000g
Magnesium sulphate	0.500g
Rose Bengal	0.050g
Agar	15.000g
Final pH (at 25°C)	7.2±0.2

2.2.2 Reagents

Lactophenol Blue:

This is mainly used as a staining solution for yeast or fungi.

Composition of Media:

Table 07: Composition of Lactophenol Blue Medium

Ingredients	gm/L
Phenol crystals	20.0g
Cotton blue	0.050g
Lactic acid	20.0ml
Glycerol	20.0ml
Distilled water	20.0ml

*Media components information has been taken from bottle label tags and online technical data base of HIMEDIA.

2.3 Appearances of *C. zeylanoides* in Different Media:

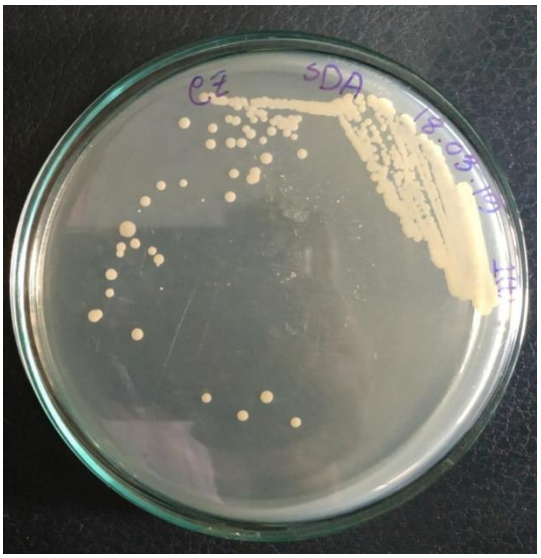

Fig 01: Growth on SDA agar plate

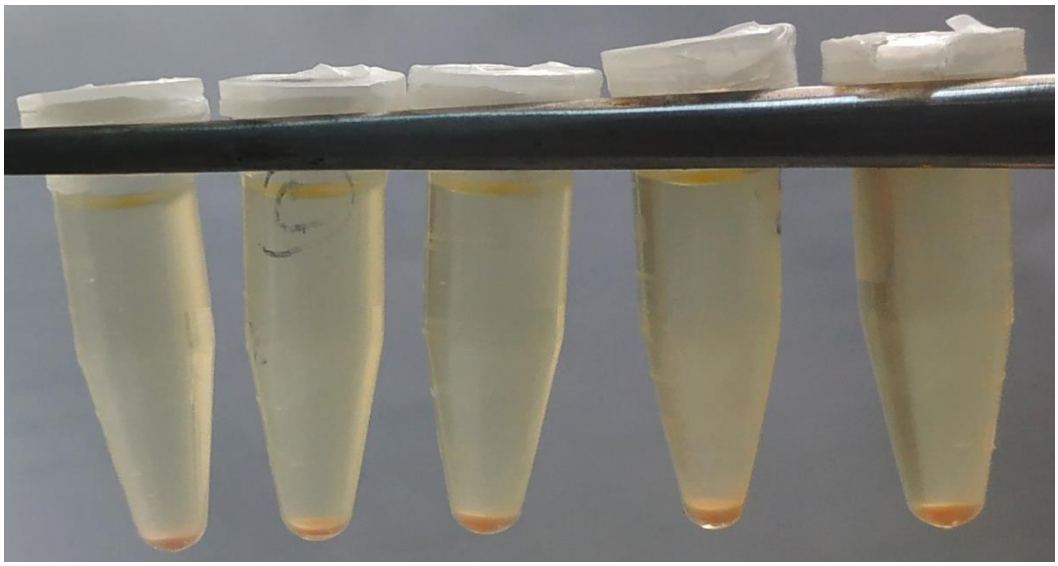

Fig 02: Growth in SDB broth

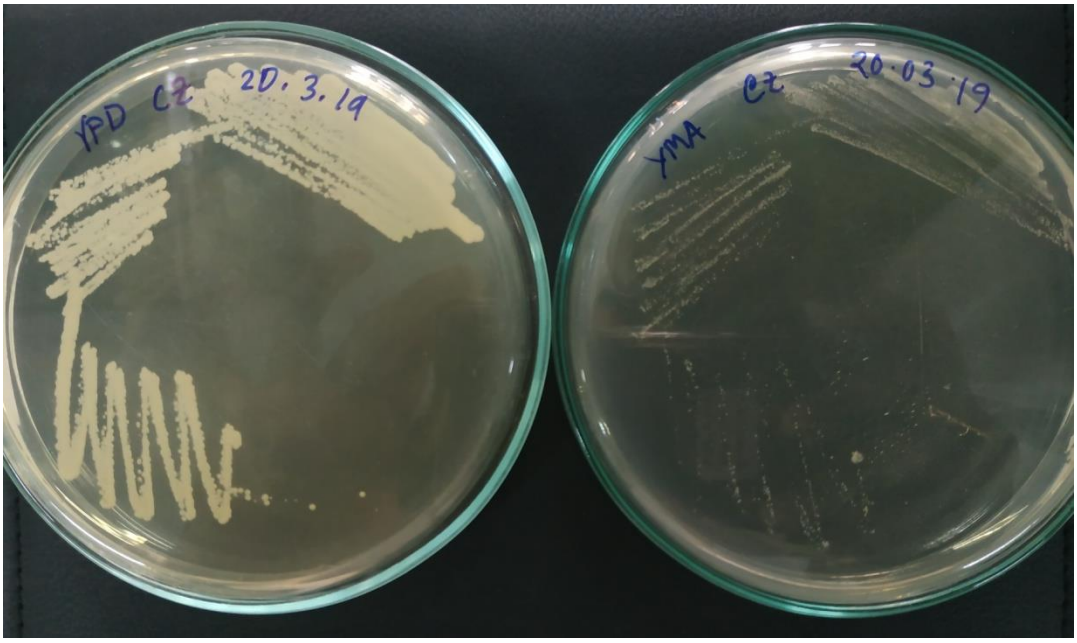

Fig 03: Growth on YPD agar plate (left side) and YMA agar plate (Right side)

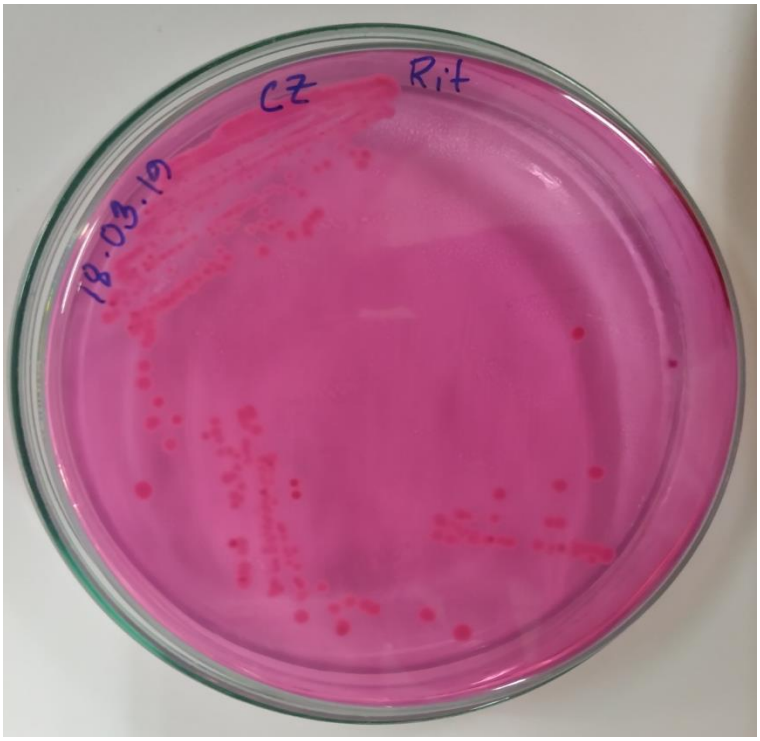

Fig 04: Growth on Rose Bengal agar plate

2.4 Congenial Media

Among the other media like YPD, YMA, Rose Bengal Agar; SDA and SDB came out as the appropriate media selected for the optimum growth of *Candida zeylanoides* as the growth was better in both Sabouraud Dextrose Agar and Broth. Compared to this, in other media, the growth of *Candida zeylanoides* was slower.

2.5 Equipment Glassware:

- Petri dishes
- Glass bottles
- Eppendorf Tubes
- Slides & Cover Slips
- Incubators
- Test tubes
- Bikers
- Conical Flasks
- Microscope
- Weighing Balance
- Micropipette
- Autoclave
- Laminar Flow Cabinets
- Spectrophotometer
- Para film
- Biolog Microbial Identification system

2.6 Methods

Determination of Heat Stress:

The heat stress test was performed to check the heat variance absorbing capacity of *C. zeylanoides.* Yeast culture was sub-cultured on SDA plates to isolate the pure colonies for 24 hrs at 32.5° C. The culture was arranged as in the dilution factors of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} times and left for the incubation for 24 hrs again at 32.5° C by arranging them in according to the time intervals. After passing the incubation period the absorbance was taken at 600 nm. The absorbance was taken by holding down the time intervals of 0 min, 60 mins, 120 mins, 180 mins, 240 mins and 300 mins. To trace the tolerance ability of the yeast cells morphological appearances were been checked.

Bile Salt Tolerance:

C. zeylanoides culture was subcultured in SDA plates for fresh cultures. To get the accurate result and experiences the right changes the solution was needed to be freshly made in the lab for the experiment. No pre-prepared bile salt solution could be used for this. Afterward, the single colony was taken and added to the different concentrations of freshly made bile salt solutions (0.1%, 0.5% and 1%) and mixed them by vortexing mildly. They were set in the Eppendorf tubes and the time intervals were mentioned on it to ease the measuring absorbance. These were incubated for 24 hrs at 32.5° C. After 24 hrs those Eppendorf tubes were taken out and by diluting them in a manner of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilution factors and were placed on SDA plates according to their dilution factors and interval times incubated at 32.5° C for 24 hrs. In the meantime, the absorbances of the bile salt cultures were measured at 600 nm by maintaining the time intervals. Then after 24 hrs of incubation, the growth of the *C. zeylanoides* yeast cells was estimated after 0 min, 60 mins and 120 mins of incubation at 32.5º C by measuring the absorbance at 600 nm and the number of viable cells was determined by plate count method (colony counter) was measured further after 24 hrs by maintaining the time intervals of 0 min, 60 mins and 120 mins respectively and the result expressed as percentage log survival was calculated.

Tolerance to Acidity:

C. zeylanoides yeast cultures were activated in SDA agar plates and incubated at 32.5º C for 24 hrs. The acidity tolerance test was performed in artificial lab gastric juice which was made in the lab for this experiment. Like the bile salt, the gastric juice was also freshly made as the experiment need to be performed in freshly made gastric juice to get the appropriate result of it. In SDA plates the culture of *C. zeylanoides* was sub structured for fresh cultures. The approach needs to be made in the laboratory for the patient to get the exact results and undergo the right changes. There could be no pre-prepared salt bile solution. Subsequently, the individual colonies were taken and applied to varying amounts of freshly made gastric juice solutions (3, 3.5, 4 and 4.5 strength of acidity) and slightly blended. They were put in the Eppendorf tubes and the measurement absorption time intervals were described. This was incubated at 32.5° C for 24 hrs. These solutions were diluted after 24 hrs and diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} factors of dilution, and spread on SDA plates and incubate at 32.5° C for 24 hrs according to their dilution factors and time interval. Then, the absorbance was measured by maintaining time intervals of gastric juice cultures were measured at 600 nm. After 24 hours of incubation, the growth of the *C. zeylanoides* yeast cells at 32.5^{o} C was estimated at different time intervals of 0 min, 60 mins and 120 mins of incubation by measuring absorbance at 660 nm and the number of viable cells was calculated using a plate counting method (colony counter) after 24 hrs to 0 minutes, 60 minutes and 120 minutes of incubation respectively and the result was expressed as a percentage log survival.

Osmotic Stress Tolerance Test:

Freshly made dextrose and sucrose of five different concentrations were made for the observance of osmotic stress levels in the cells of *C. zeylanoides.* The subculture of *C. zeylanoides* was inoculated from the SDA plate to the 1x, 3x, 5x, 7x and 9x sucrose and dextrose broth respectively by diluting them up to fourth time in a manner of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilution factors. Set for incubation at 32.5° C for 24 hrs by grouping them in time intervals. The

absorbance was measured at 600 nm after the incubation time period expired. Absorbances were taken by holding down 0 min, 60 mins, 120 mins, 180 mins, 240 mins and 300 mins time intervals. Morphological appearances were observed to map the resistance ability of the yeast cells and cell shapes were identified under light microscopic.

2.7 Influence of Physiochemical Parameters on the Growth of Isolates

2.7.1 Effect of Temperature

To study the effect of temperature on the growth of *C. zeylanoides* and organisms were cultured at different temperatures. The growth pattern was observed by measuring absorbance at 600 nm at different time intervals using a spectrophotometer.

The isolated organism was inoculated into SDB broth and incubated overnight (32.5° C). Fresh SDB broth was transferred to seven sets of Eppendorf tubes for culturing the organisms at seven different temperatures. For each organism, 200 µl of sample suspension was inoculated into 1000 µl fresh broth in each Eppendorf tube. The Eppendorf tubes were incubated at different temperatures (20° C, 25° C, 30° C, 32.5° C, 37° C, 40° C and 45° C). The control tubes were left un-inoculated. After 24 hrs, cell density was determined by measuring absorbance at 600 nm using a spectrophotometer.

2.7.2 Effect of pH

Organisms were grown at different pH to study the effect of pH on the growth of isolates. The growth pattern was detected by measuring absorbance at 600 nm at different intervals of time using a spectrophotometer.

They inoculated sample specimens in nutrient broth and incubated at 32.5° C overnight. Flasks with a fresh SDB broth containing optimal substrate concentration and pH adjustments were made to 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 in different flasks using HCl (to increase acidity) and KOH (to increase alkalinity). Flasks were then autoclaved. The subcultures were inoculated and

incubated for 24 hrs at 32.5° C. At the end of the incubation period, cell density was measured using a spectrophotometer at 600 nm.

2.7.3 Effect of Salinity

C. zeylanoides was cultured at different saline concentrations of NaCl to study the effect of saline concentration on yeast organism strain production. The growth trend was detected by using a spectrophotometer to measure the absorbance at 600 nm at different time intervals.

Inoculated in the nutrient broth, *C. zeylanoides* was incubated overnight at 32.4 °C. Flasks with fresh SDB broth containing maximum concentration and salinity gradually increased to 1%, 2%, 3%, 4%, 5%, 6%, and 7% in separate flasks by introducing sodium chloride (NaCl). The culture was inoculated in the mentioned process and incubated for 24 hours at 32.5 ° C. At the end of the incubation period, cell density was measured using a spectrophotometer to measure the absorbance at 600 nm.

2.8 Statistical Analysis:

All experiments were carried out in triplicates. Data were presented as the mean ± standard deviation (SD) for the indicated number of independency performed experiments.

Chapter 3

Results

Chapter 3

Results

3.1 Identification of Yeast Strains

Following some steps the identification of the strain of the yeast was done. The series of the procedure that had been pursued in the identification process are given below:

Step 1. Culture Isolation on Biolog Recommended Media

• A pure culture on agar media was isolated.

• Biolog recommended media (BUY Agar) was used and incubated at 26-28°C. All species that could be identified with the YT MicroPlate would grow under these conditions.

Step 2. Specimen Preparation and Characterization

• A Wet prep or Gram stain was performed if necessary to verify that it was a yeast.

• The yeast was growing using the recommended conditions. The choice of the agar medium was very important since it must support growth and promote retention of full metabolic activity to accurately match the metabolic patterns in the YT database. Biolog YT Micro Plate 3.

• The cells must be freshly grown since many strains lose viability and metabolic vigor in stationary phase. The recommended incubation period for most organisms is 24 to 48 hours.

• If insufficient growth was obtained to inoculate the panel, ~~inoculate~~ more than one agar plate was inoculated and Incubate 24-48 hours.

Step 3. Inoculum Preparation

•The establishment of the acceptable turbidity range on the turbidimeter. At first, the 100% transmittance adjustment was done using a clean uninoculated water tube was set. Then, the

desired turbidity was determined with the YT Turbidity Standard. By using the Biolog turbidimeter and 20 mm diameter tubes, this should give a transmittance level of about 47%. These readings might vary slightly on different Biolog Turbidimeters. With other instruments or with other tubes, the transmittance readings might vary substantially.

• The turbidimeter (transmittance = 100%) was blanked with a clean tube containing un-inoculated water. Because the tubes that were used were not optically uniform, they should be blanked individually and not rotated in the light path of the turbidimeter.

• A uniform suspension was prepared as follows: Cells were removed from the agar plate with a sterile cotton swab so as not to carry over any nutrients from the agar medium into the suspension. Started with isolated colonies and then went into areas of heavier growth if necessary. Was twirled and pressed the swab against the inside surface of the tube on the dry glass above the fluid line to break up clumps and release cells. The fluid was then become a homogenous, clump-free suspension. A sterile transfer pipette might also be used to mix the suspension without creating an aerosol. If clumps were present, the tube was left to stand for several minutes and allow them to settle to the bottom.

• The density was adjusted. The meter needle went toward the acceptable turbidity range was watched. The acceptable turbidity range was defined as by the turbidity standard plus or minus 2% transmittance. This must be done with precision since it establishes the oxygen concentration for the cells and for the redox chemistry. The density could be lowered by adding more water or raised by adding more cells.

• The inoculation of the cell suspension into the MicroPlate was done promptly. Some strains lose metabolic activity if held too long (more than 20 minutes) in water without nutrients.

Step 4. Inoculation of the MicroPlate

• The MicroPlate was labeled with the organism name/number.

• The cell suspension was poured into the multichannel pipette reservoir.

• 8 sterile tips were fastening securely onto the 8-Channel Repeating Pipettor. The process was referred to manufacturer's instructions.

• The tips were filled and checked to see that all tips are filling equally. If not, refasten was done to any loose tips.

• The tips should be primed if using a manual pipettor by dispensing the first delivery back into the reservoir. The electronic pipettor performs priming automatically.

• All the wells should be filled with 100 µl. One should be careful not to carry over chemicals or splash from one well into another.

• The MicroPlate should be covered with its lid.

Step 5. Incubation

• The incubation of MicroPlate should be done at 26-28°C.

• A source of moisture should be provided in the incubator to help minimize the dehydration of the outer wells of the MicroPlate. The placing of the MicroPlates in a plastic container with wet paper towels on the bottom should be sufficient.

• The plates should be placed for 24, 48 or 72 hours, until a sufficient pattern is formed.

Program	MicroLog 3/5.2.01 35
Project	ML5
File Name	30.01.19.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	Li 1
Sample Source	
Operator	
Microplate Lot No	3005121
IF Lot No	
Comments	
Date & Time of Read	Jan 30 2019 12:10 PM
Biolog ID DB	Yt60172.eid

Result	Species ID: Candida zeylanoides
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.894	0.760	2.231	Yt	Candida zeylanoides
2	0.103	0.083	2.964	Yt	Candida catenulata
3	0.003	0.002	4.201	Yt	Candida santamariae
4	0.000	0.000	5.000	Yt	Rhodotorula hylophila

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	19	{ 37	12	< 201	5 +	< 324	< 291	< 130	< 183	{ 77	{ 61
B	19	{ 66	{ 45	{ 31	14	13	1	6	11	{ 33	{ 58	4
C	< 89	< 126	< 108	{ 44	13	{ 55	< 150	< 100	< 128	{ 26	17 +	< 95
D	0	{ 56	< 88	11	6	< 90	{ 60	{ 63	< 127	{ 75	{ 62	{ 48
E	{ 27	{ 40	10	14	{ 32	15	5	10	5	{ 25	{ 35	-1
F	{ 45	{ 30	< 112	{ 44	{ 28	15	1	9	24	7	23	19
G	-2	{ 57	{ 51	{ 52	{ 42	{ 42	-1	23	11	15	18	7
H	-17	{ 25	3	{ 60	{ 52	{ 53	23	5	{ 56	{ 28	{ 33	-5

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Fig 05: Identification of *C. zeylanoides* by Biolog™ system

The isolation and identification part of *C. zeylanoides* was done by the scientific officers of the Industrial Microbiology lab, IFST, BCISR. Under their supervision and technical appliances the whole process of isolation and identification of *C. zeylanoides* was done.

3.2 Optimum Growth Temperature Test

To detect the optimum temperature of the strain of *C. zeylanoides* the yeast cultures were incubated at seven different temperatures of 20°C, 25°C, 30°C, 32.5°C, 37°C, 40°C and 45°C for 24 hrs. It was observed that the optimum growth temperature of *C. zeylanoides* was 32.5°C (Fig. 06)

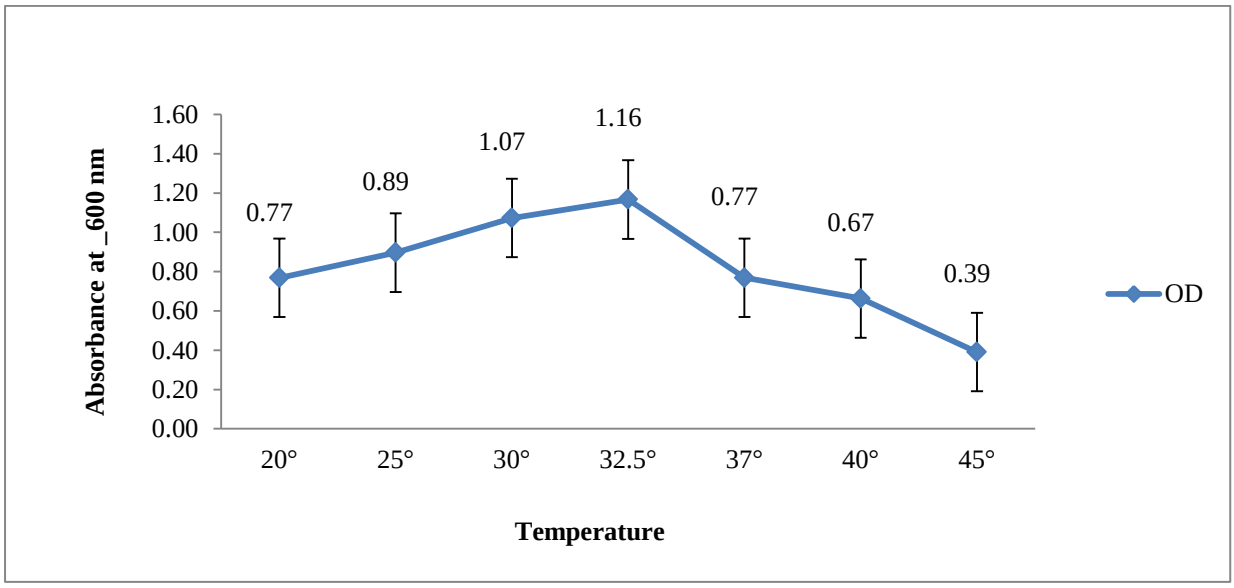

Fig 06: Optimum growth temperature of *C. zeylanoides* after 24 hrs

3.3 Salinity Tolerance Test

To know the tolerance of salinity of *C. zeylanoides* flasks contained SDB broth with the maximum concentration of substratum and salinity gradually increased to 1%, 2%, 3%, 4%, 5%, 6%, and 7%. This test was proceeded to detect the salinity tolerance level. Here the growth was going quite fine from 1% to 3% of NaCl. Fig. 07 shows the tolerance level decreased afterward as the salinity levels were increasing.

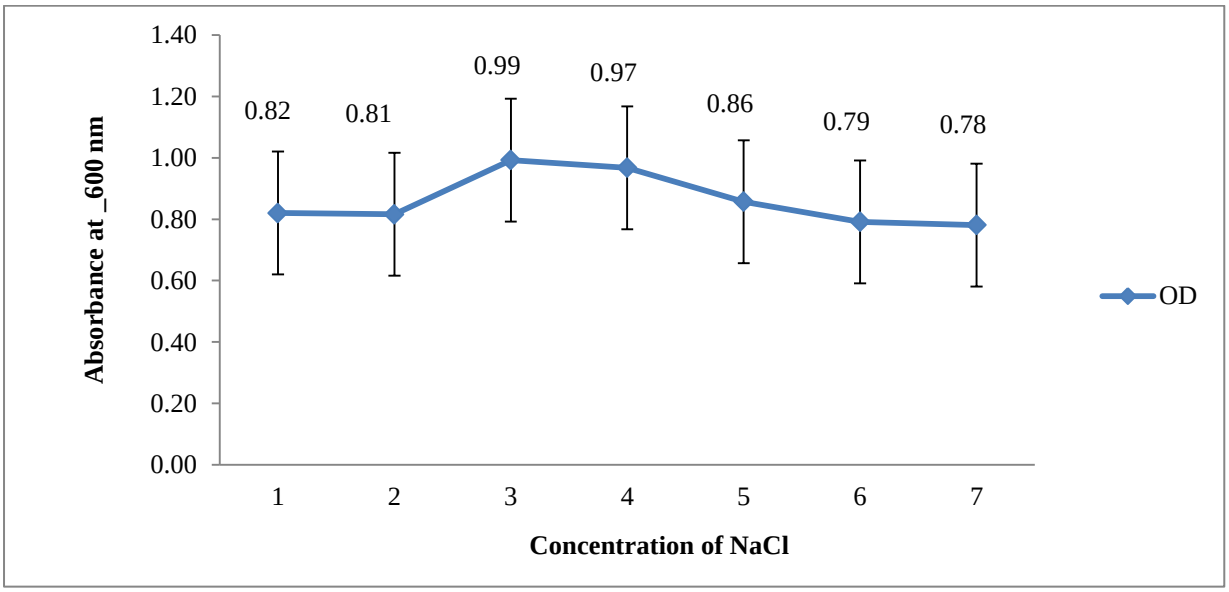

Fig 07: Salinity tolerance level of *C. zeylanoides* after 24 hrs

3.4 pH Level Identification Test

The pH of the medium was adjusted to 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5. This test was done to determine the fortitude capacity of *C. zeylanoides.* This pH test result was showing that the acid tolerance of this strain was as fine as the enduring level of alkalinity (Fig. 08).

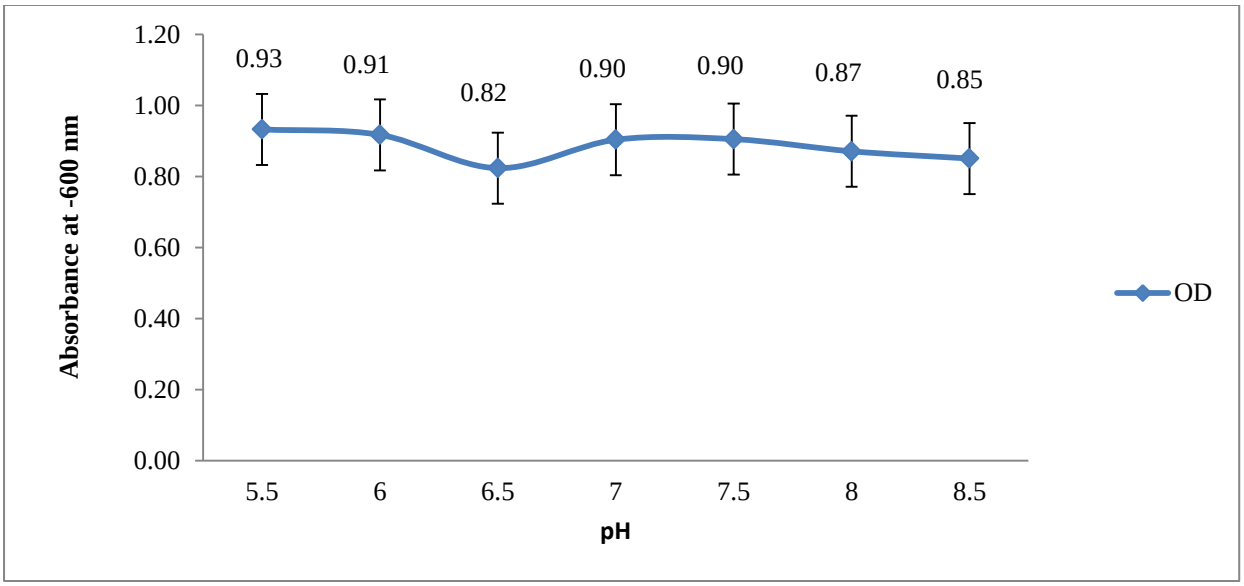

Fig 08: pH level of *C. zeylanoides* at 24 hrs of incubation

3.5 Heat Stress Tolerance Test

This experiment was done to discuss the optimum growth flexibility and cell physiognomy of *C. zeylanoides* at certain temperature levels. Six segments of time intervals were set to measure the absorbance differences and cell physiognomy of the cultures. At the end of the incubation period, cell density was measured using a spectrophotometer at 600 nm. At temperature 30° C to 37° C, the growth was fine and the cells were enduring the changing of temperature in a quiet fine manner. But when the temperature reaches 40° C the cell growth got slowed down and at 44°C the growth was less than other temperatures and the cells got ruptured extensively. The experiment was done in three-time segments of 24 hrs, 48 hrs and 72 hrs. The growths were subtle in 24 hrs and then for growing more over 48 hrs the cell quantity increases and in 72 hrs the growth slowed down because of excessive cell growth.

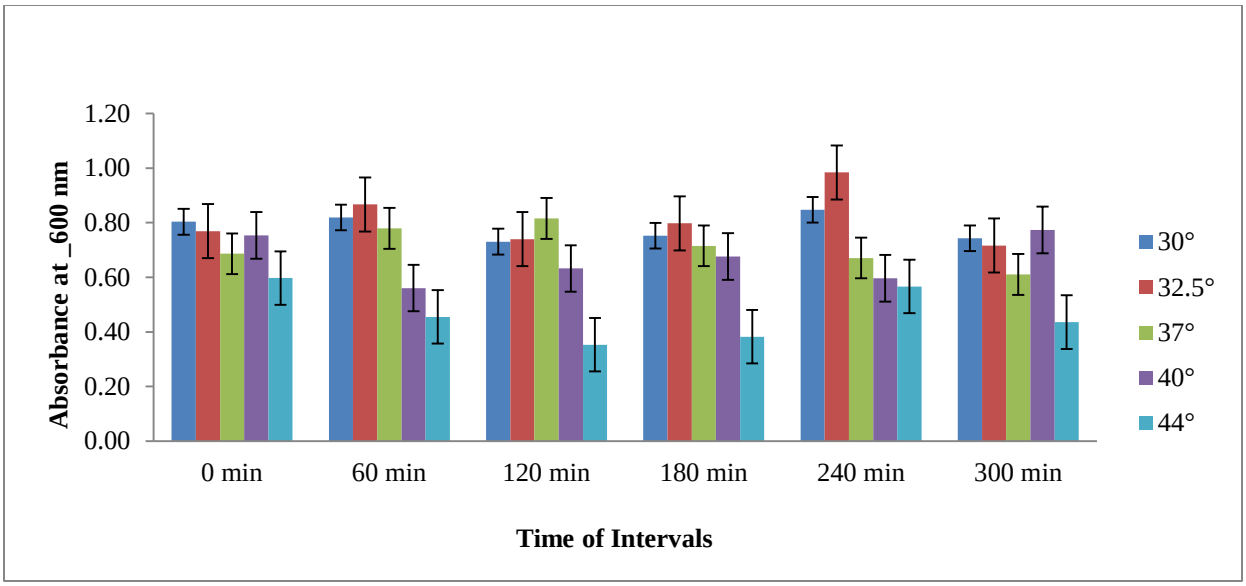

Fig 09: Heat stress detection of *C. zeylanoides* at different time intervals after growth for 24 hrs

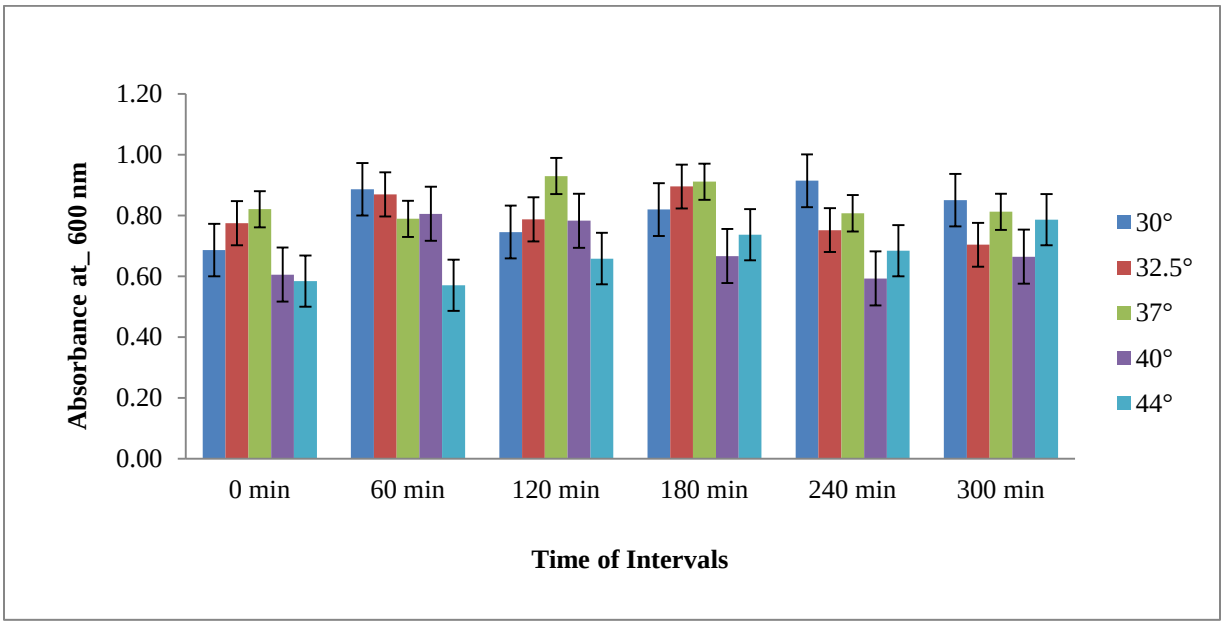

Fig 10: Heat stress detection of *C. zeylanoides* at different time intervals after growth for 48 hrs

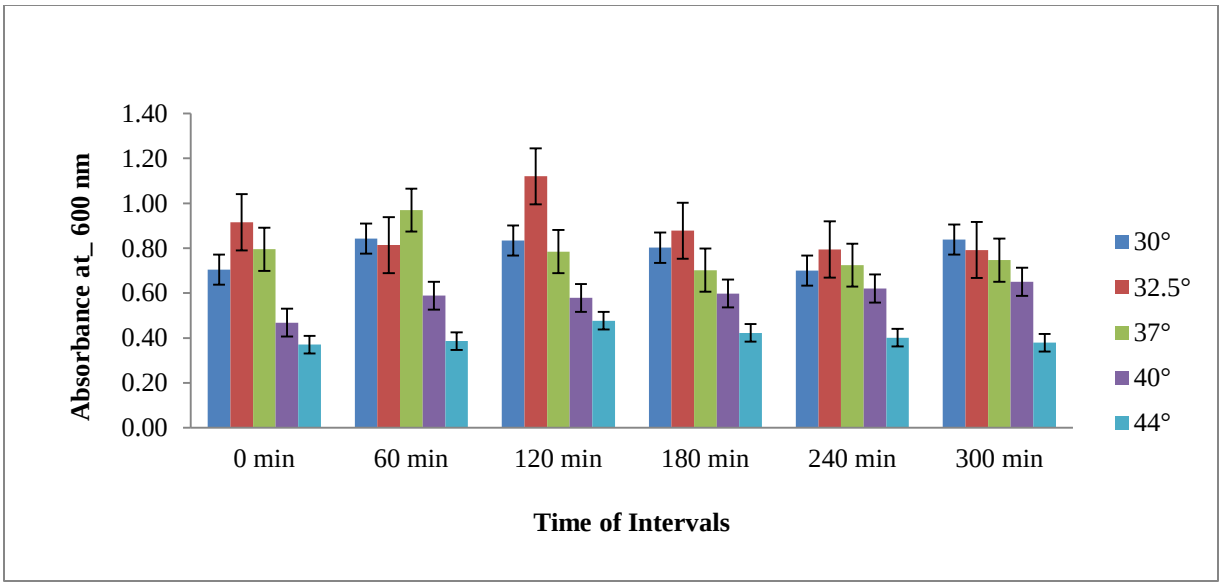

Fig 11: Heat stress detection of *C. zeylanoides* at different time intervals after growth for 72 hrs

3.5.1 Morphological Representation

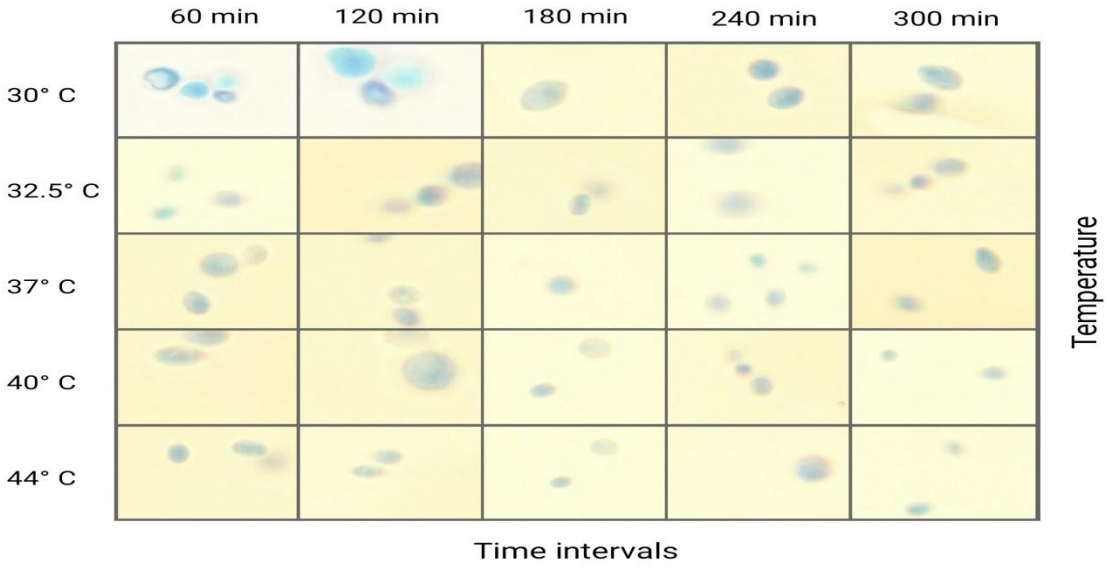

Fig 12: Morphological representation of *C. zeylanoides* at different time intervals after 24 hrs for Heat stress

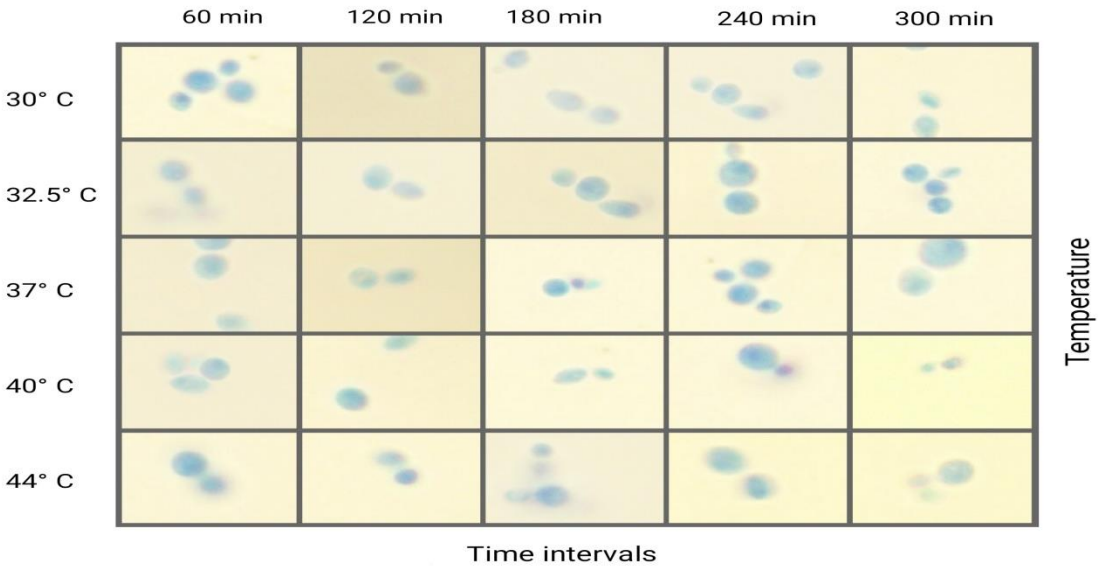

Fig 13: Morphological representation of *C. zeylanoides* at different time intervals after 48 hrs for Heat stress

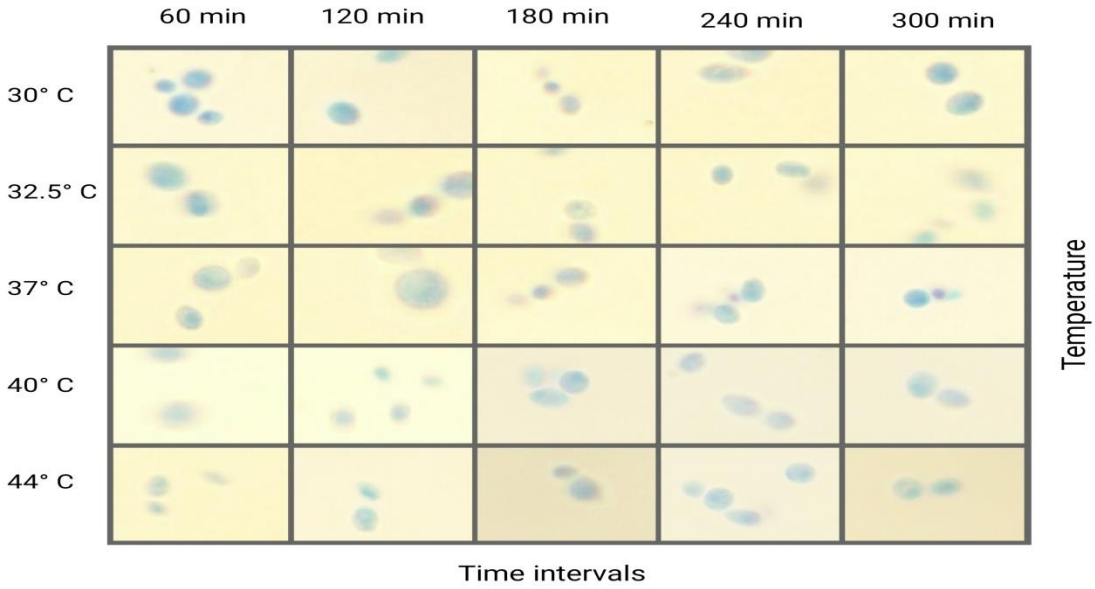

Fig 14: Morphological representation of *C. zeylanoides* at different time intervals after 72 hrs for Heat stress

3.5.2 CFU Count for Heat Stress Test

Table 08: Colony count of *Candida zeylanoides* after incubation for different time intervals

Temperature	24 hrs/CFU	48 hrs/CFU	72 hrs/CFU
30° C	TNTC	TNTC	TNTC
32.5° C	TNTC	3.12×10^{6}	TNTC
37° C	1.89×10^{6}	1.99×10^{6}	1.68×10^{6}
40° C	1.45×10^{6}	1.78×10^{6}	1.13×10^{6}
44° C	No Growth	No Growth	No Growth

3.6 Osmotic Stress Tolerance Test:

This test was done to detect how the cells respond towards certain concentrations of solutions and the cell's morphological changes to the concentration pressure. Here dextrose and sucrose sugar solution of five different concentrations (1x, 3x, 5x, 7x and 9x) levels had been used. Cell density was measured using a spectrophotometer to measure the absorbance at 600 nm and the cell's morphology was observed. In dextrose solution, the growth was maximum at 7x concentration level and then the tolerance level had been dropped at 9 x levels. On the other hand, the growth level was fine at 5x level in the case of sucrose and then gradually decreased.

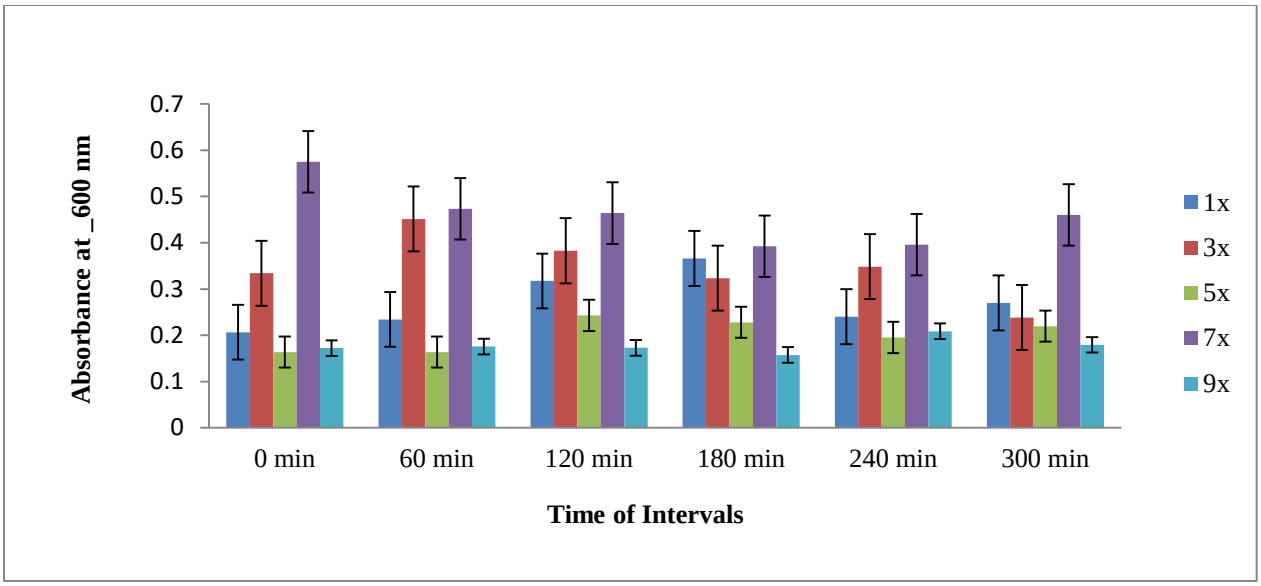

Fig 15: Osmotic stress level of *C. zeylanoides* at different dextrose concentrations and at different time intervals after growth for 24 hrs

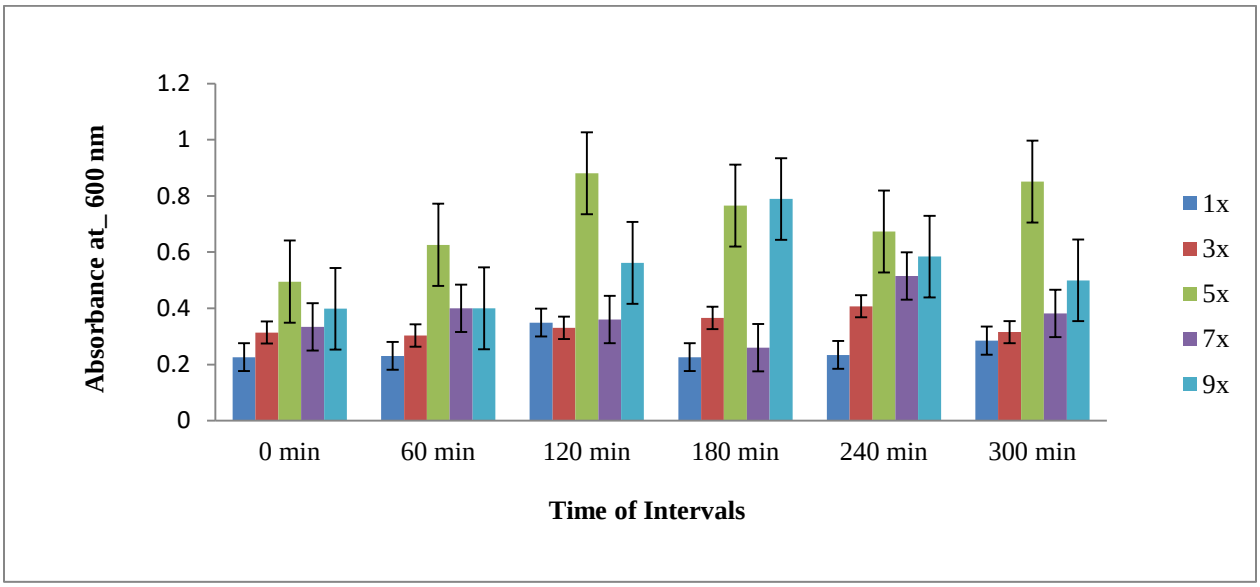

Fig 16: Osmotic stress level of *C. zeylanoides* at different sucrose concentrations and at different time intervals after growth for 24 hrs

3.6.1 Morphological Representation

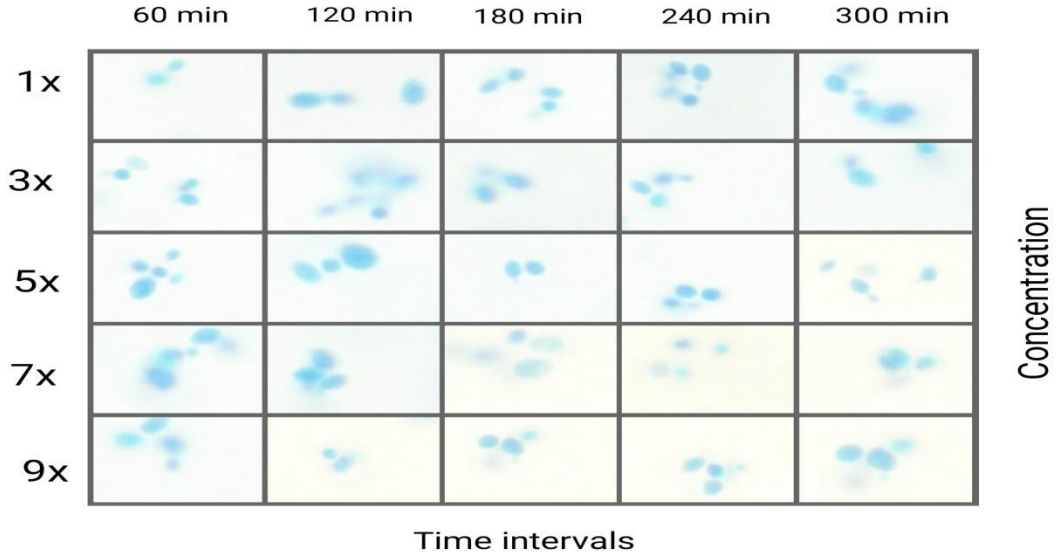

Fig 17: Morphological representation of *C. zeylanoides* at different time intervals and different concentrations of dextrose after growing for 24 hrs

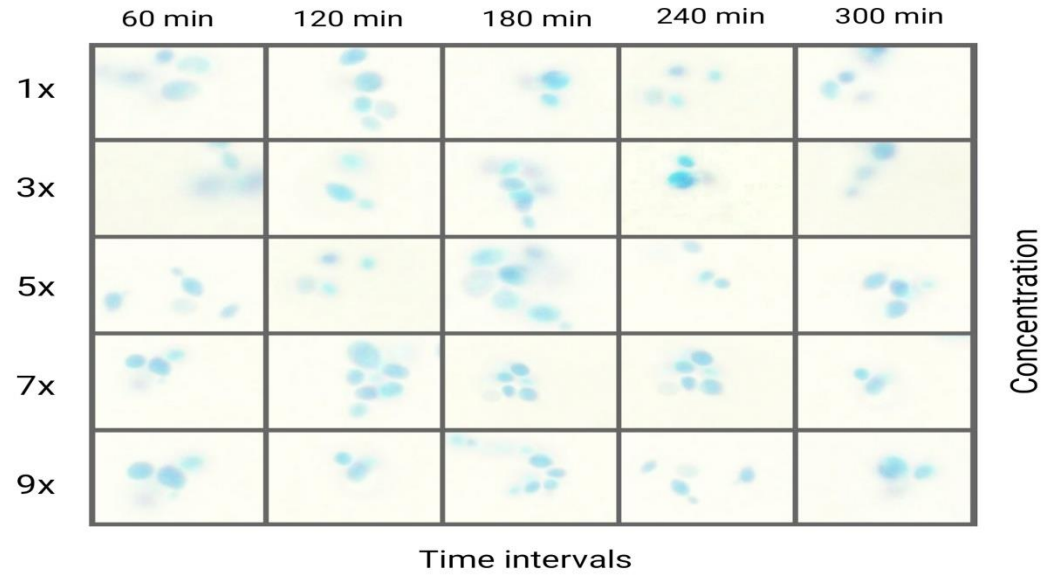

Fig 18: Morphological representation of *C. zeylanoides* at different time intervals and different concentrations of sucrose after growing for 24 hrs

3.6.2 CFU Count for Osmotic Stress Test

Table 09: Colony count of *C. zeylanoides* at different concentration of dextrose and at different time intervals after growing 24 hrs

Concentration Of Dextrose	60 min/CFU	120 min/CFU	180 min/CFU	240 min/CFU	300 min/CFU
1 x	3.00×10^{6}	1.72×10^{6}	3.84×10^{6}	6.00×10^{5}	3.84×10^{6}
3 x	6.60×10^{5}	2.08×10^{6}	6.40×10^{5}	6.30×10^{5}	4.80×10^{5}
5 x	1.63×10^{6}	1.28×10^{6}	3.64×10^{6}	7.10×10^{5}	4.70×10^{5}
7 x	3.36×10^{6}	4.12×10^{6}	3.84×10^{6}	1.05×10^{6}	4.00×10^{5}
9 x	1.32×10^{6}	1.48×10^{6}	3.36×10^{6}	4.30×10^{5}	2.48×10^{6}

Table 10: Colony count of *C. zeylanoides* at different concentration of sucrose and at different time intervals after growing 24 hrs

Concentration Of Sucrose	60 min/CFU	120 min/CFU	180 min/CFU	240 min/CFU	300 min/CFU
1 x	3.96×10^{6}	7.44×10^{6}	1.28×10^{6}	4.60×10^{5}	2.84×10^{6}
3 x	8.00×10^{5}	1.24×10^{6}	***3.48×10^{6}**	1.27×10^{6}	2.56×10^{6}
5 x	2.72×10^{6}	***3.48×10^{6}**	1.76×10^{6}	7.00×10^{5}	3.08×10^{6}
7 x	5.40×10^{5}	2.92×10^{6}	4.40×10^{5}	1.00×10^{6}	1.72×10^{6}
9 x	2.24×10^{6}	2.04×10^{6}	2.60×10^{5}	1.20×10^{6}	2.89×10^{6}

*****Maximum count

3.7 Probiotic Activity Test:

To ascertain the probiotic activity gastric juice and bile salt tolerance test was done. The test showed that the growth of *C. zeylanoides* or the value of absorbance decreased with the increase of salt concentration from 0.1% to 1%. In the case of the gastric juice test result, the cells can tolerate pH up to 4.5 which means *C. zeylanoides* may have the probiotic activity strain in it.

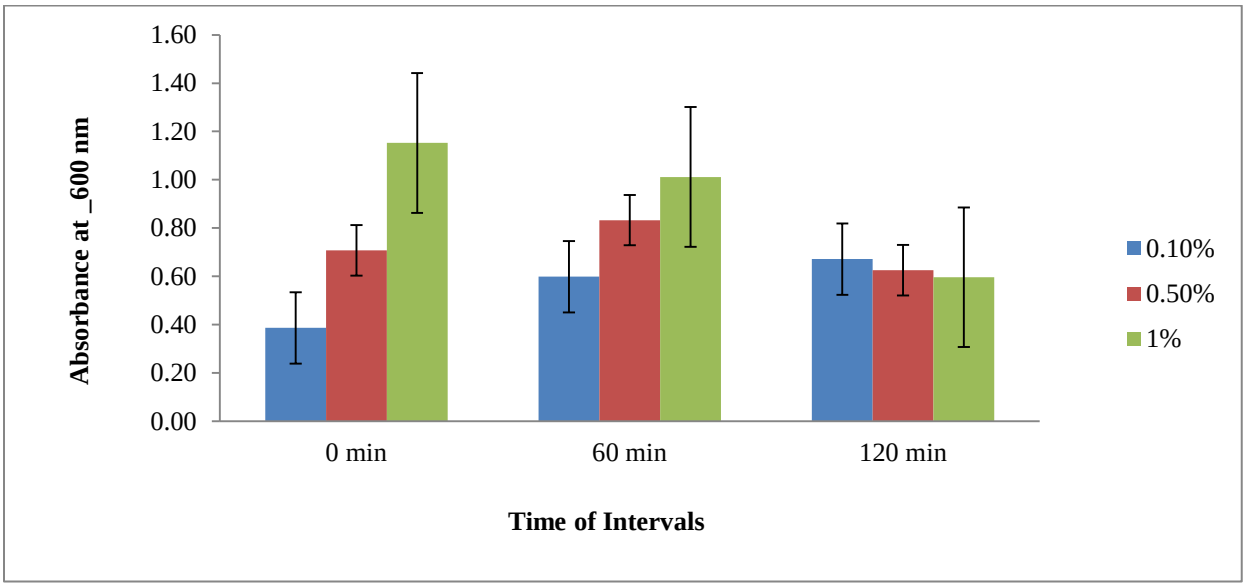

Fig 19: Probiotic activity level of *C. zeylanoides* at different bile salt concentrations and at different time intervals after 24 hrs

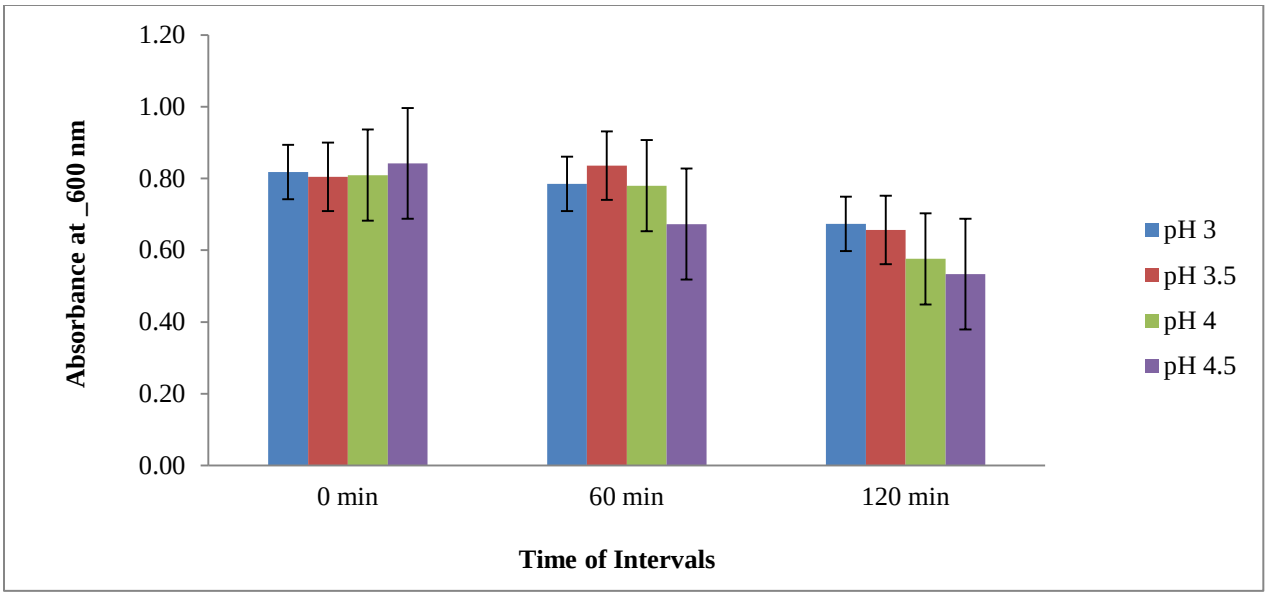

Fig 20: Probiotic activity level of *C. zeylanoides* at different gastric juice concentrations and at different time intervals after 24 hrs

Chapter 4

Discussions

Chapter 4

Discussion

In this study, the yeast strain *C. zeylanoides* were collected from the local cheese. It was isolated and the original strain was identified. This cheese has uniqueness in its appearance as it has a pink pigment. Other than its physiognomy, this study deals with the tests that had been performed to ascertain some of this strain's cabalistic competences on various sections like; its isolation, characterization, its caliber to endure heat stress, its efficiency to undergo such osmotic stress and the probiotic efficacy of it.

Temperature is an emergent fact that should be in consideration in case of understanding any organism and its growth competence. Apprehend the optimum temperature for the growth of *C. zeylanoides* was come out as 32.5° C. In seven different temperatures (20° C, 25° C, 30° C, 32.5° C, 37° C, 40° C and 45° C) in which the growth of yeast was tested to know the optimum temperature level of *C. zeylanoides*. In all the seven temperature (20° C, 25° C, 30° C, 32.5° C, 37° C, 40° C and 45° C) this strain grew well but there was certainly growth differences among them. Growth was severely retarded after at 40° C or above that. The reading was taken after 24 hrs. On the other hand, the optimum growth temperature of *Saccharomyces cerevisiae var. boulardii* is 37° C (Łukaszewicz, 2012). *Saccharomyces cerevisiae* is the yeast best adapted to grow at high temperatures within the *Saccharomyces* genus, with the highest optimum (32.3° C) and maximum (45.4° C) growth temperatures (Salvadó *et al*., 2011). Skim through the temperatures of other yeast strains these chosen seven temperature parameters were taken to identify the suitable temperature level for the growth of *C. zeylanoides.* In the level of 32.5° C the growth came out in an ample number that helped to determine 32.5° C as its optimum temperature.

The heat stress fortitude tendency of *C. zeylanoides* was checked several temperatures (30° C, 32.5° C, 37° C, 40° C and 44° C) at 24 hrs. In all the first levels of temperature, growths were fine (up to 37° C) however the growth becomes a little less at 40° C and very little when the temperature reached 44° C. At some point, there was also no growth shown in the culture plate.

But the heat stress enduring test helped to show the pattern of cell growth along with the temperature tolerance capacity of *C. zeylanoides.* The optimal growth temperature for *C. zeylanoides* was assessed through the measurement of OD_{600} and by counting the CFUs up to 300 min.

Osmotic pressure is related to the cell permeability to any solution. The cells of an organism should be enough capable to endure stress levels of osmotic solution pressure. Due to up taking a different concentration of solutions, the shapes and structures of the cell might get changed. In the case of *C. zeylanoides* , the cells were going well in their lag phases up to concentration level by 1x, 3x and 5x concentration of dextrose but the growth undergo the level when the concentration becomes higher like of 7x and 9x. The cell structure and permeability of *C. zeylanoides* were assessed through the measurement of OD_{600} and by counting the CFUs up to 300 min after growth for 24 hrs. The cell growth of *C. zeylanoides* was fine in both dextrose and sucrose in terms of osmotic pressure test but at the onward stage of the test when the concentration becomes higher, the growths were started to reduce and cells showed a faint color which indicates the cells might get ruptured a bit due to the high concentration. The cell shapes and structures were also changed.

Probiotics are classified as living micro-organisms that provide the host with a health benefit (Czerucka, *et al.,* 2007). To destine whether an organism has probiotic activity or not, several tests should be performed. In the lumen of the human stomach, the pH of gastric acid is 1.5 to 3.5. Due to the test of bile salt tolerance and gastric juice tolerance test *C. zeylanoides* survived well. Therefore to have the probiotic activity, tolerance to gastric juice is very important to have and *C. zeylanoides* have the endurance from pH level 3 to 4.5. But more work should be done on this site to explore more about its probiotic activity.

Conclusion:

Yeasts have a vast area to study and to work on. A variety of yeasts are there that have a cabalistic contribution in many sectors like bakery, aromatherapy, wine production, fermented food production, etc. Yeasts are temperature sensitive so that they needed to be handled in a very outgiving situation to explore the best sides of these. Many pieces of research have been taken

place on yeast to know more about their efficacies. *Saccharomyces cerevisiae is* the most common yeast strain to work on. It's used to call the sugar fungi. Many members of this genus are considered very important in food production. It is known as the brewer's yeast or baker's yeast. On the other hand, *C. zeylanoides* is a new strain and very less amount of work has been done on it. Though from this study its isolation, characterization, its caliber to endure heat stress, its efficiency to undergo such osmotic stress and the probiotic efficacy of it had been portrait a bit; yet much work opportunity is there to know more about it. In the future, there might be some work that will take place to identify its probiotic activity more meticulously apropos their flourishing sides in the medical and medicinal sector.

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