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Effect of Physico-Chemical Parameters in the Production of Hydrolytic Enzymes from Yeast *Candida Tropicalis* Isolated from the Mangrove Sediments of North Kerala, India

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Abstract

The extracellular enzymes produced by yeasts present in mangrove sediments were found to have wide range of biotechnological and industrial applications. Large scale production of these enzymes by bioprocess techniques needs proper optimization of culture conditions for high enzyme yield in a cost-effective manner. In the present study, we investigated the effect of different growth conditions like pH, salinity, substrate concentration and temperature on the growth and production of enzymes protease, amylase, lipase and ligninase by selected strains of *Candida tropicalis*. The optimum conditions for the maximum growth and enzyme production for protease, amylase, lipase and ligninase producing strains were found to be at pH 8.5-9; salinity 5-10 ppt and temperature 35 – 40°C with 2% casein, 1% starch, 1% tributyrin, 0.5% tannic acid as substrate concentrations respectively. The results suggest the use of yeast *C. tropicalis* from mangrove sediments as potent and promising strain for the large-scale production of hydrolytic enzymes when compared to the previous studies done on marine counter parts.

Keywords: Mangrove sediments, yeasts, *Candida tropicalis,* extracellular enzymes, optimization, culture conditions

1. Introduction

Benthic yeasts from mangroves have wide ecological significance as they are involved in various transformation processes. This is attributed by the extracellular enzymes produced as a result of different metabolic reactions that take place inside their cell (Kutty *et al*., 2014). Yeast enzymes were found to be actively involved in nutrient recycling, decomposition of litter, mineralization of organic compounds and degradation of oil and recalcitrant substances (Kutty *et al*., 2012; Pothayi and Devasia, 2020). Moreover, the benthic fauna of mangroves survives in extreme conditions such as high salinity variation, fluctuating temperature, low oxygen concentrations, high UV exposure and varying nutrient compositions. The secondary metabolites from mangroves which are toxic to microorganisms are detoxified or degraded by them as a part of their metabolic activities. These factors act as driving force for the microbes to compete with each other and evolve to produce novel bioactive compounds (Solntsev *et al*., 2019). So, the enzymes produced by them possess unique properties like salt tolerance, thermostability, anaerobic tolerance, substrate flexibility etc. (Sengupta *et al*., 2015). Altogether, these characteristics of yeast enzymes make them potential candidates for various industrial applications.

Yeast enzymes (due to their industrial, medical and food applications) have been produced in large scale recently by bioprocess technology. In comparison to other microbes, yeasts are preferred as better sources for the large scale enzyme production due to their ease in generation by environmental and genetic manipulation, simplicity of extraction, economic cost, stability, non-toxicity and quality of the metabolites (Elsanhoty *et al*., 2017). Moreover, large scale production of yeasts can be done using different types of culturemedia using cheap industrial by-products and wastes as nutrient sources (Cheng and Yang, 2016).

One of the most important yeast extracellular enzymes is lipase which is found to be produced by almost all the strains (Paskevicius, 2001). Lipases have wide catalytic activity including hydrolysis, acidolysis, esterification, alcoholysis and aminolysis (Kutty *et al*., 2014) and are used in the production of detergents, cosmetics, chemicals and pharmaceutical agents (Choudhury and Bhunia, 2015); also they have applications in bioremediation of environments contaminated with inorganic and organic pollutants, hydrocarbons and metals (Vakhlu and Kour, 2006). Yeast proteases have many significant applications in manufacture of detergents and chemicals, food and feed processing, leather and chemical industries, also in medical sector and waste treatment (Bessadok *et al*., 2015; Shahat, 2017).

Amylases that hydrolyze starch molecules are of great biotechnological importance and constitute 25% of world enzyme market. They are largely used in bread and baking industries, textile, paper, medical and detergent industries

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apart from their use in clinical biochemistry (Yalsein and Çorbaci, 2013). Their ability to convert starchy biomass into single cell proteins and ethanol has received much attention during recent years (Kachiprath *et al*., 2018). Ligninases from yeast, though least studied, are found to have applications in feed, fuel, food, agricultural, paper, textile and cosmetics industries (Bonugli-Santos *et al*., 2012).

The production of extracellular enzymes by yeasts is greatly influenced by several physico-chemical factors like temperature, pH, substrate concentration and composition, salinity, oxygen availability etc. Proper optimization of the factors affecting the growth of yeast in culture medium is of great importance for maximum enzyme production (Rahman *et al*., 2013). It would help in the large-scale production of yeast enzymes by fermentation technology in a very cost effective manner.

The main objective of the present study was to screen benthic yeast isolates from mangroves for production of lipase, protease, amylase and ligninase enzymes. Study was also focused on evaluating the effect of various physico-chemical parameters on selected strains for maximum biomass yield and enzyme production.

2. Materials and Methods

Sediment samples were collected from the mangroves of the 5 districts from 8 sites along North Kerala coast during the period 2018-2019. The sites were Chandragiri (KGD), 12°05'32" N 75°13'39" E (Kasaragod Dt), Edat (EDT), 12°05'3" N 75°13'39" E; Pazhayangadi (PYD), 12°02' 72" N 75°29'31" E; Valapattanam(VPT), 11°93'45" N 75°35'35" E (Kannur Dt), Elathur (ELR), 11°19'43" N 75°45'2" E; Kadalundi(KDI), 11°07'43" N 75°49'48" E (Kozhikode Dt), Ponnani (PON), 10°47'10" N 75°55'30" E (Malappuram Dt) and Chettuva (CTV), 11°1'41" N 75°52'62" E (Thrissur Dt).

2.1. Isolation of yeasts

Approximately, 10-20g of sub surface sediment was collected using sterile plastic corer and was transferred aseptically into sterile polythene bags. The collected samples were transported in ice boxes and processed within 2 hours of collection. For the isolation of yeasts, spread-plate method was employed using Wickerham's agar supplemented with 200 mg/L chloramphenicol (Wickerham, 1951) in duplicates. The plates were incubated at 18 ± 2° C for 7 days, and the colonies developed were purified by quadrant streaking and transferred to malt extract agar slants for further studies.

2.2. Screening for enzyme activity and Identification of potent isolates

All the isolates obtained were tested for the production of extracellular hydrolytic enzymes viz., protease, amylase, lipase, urease, ligninase, cellulase, DNase, pectinase and chitinase. Since the enzymes protease, amylase, lipase and ligninase were found to be produced by majority of the isolates, they were selected for further studies. Nutrient agar medium supplemented with casein (2%), starch (1%) and tributyrin (1%) were used for the detection of hydrolytic activities of protease, amylase and lipase, respectively. Crawford's agar supplemented with 0.5% tannic acid was used for the detection of ligninase activity. The plates were spot-inoculated and incubated at 28 ± 2º C for 7 days. Formation of clearance / halo zone around the colonies was considered as positive result for lipase. Plates were flooded with 1M HCl and Gram's iodine solution after incubation for protease and amylase, respectively and the appearance of clearance zone was noted as positive result. Formation of brown colour around the colonies was considered as positive result for ligninase. The isolates which showed maximum enzyme activity on plates at 24 – 48 hours with significant increase in their activity by 72 hours were selected for growth optimization studies.

The selected potent isolates were then characterized using morphological, biochemical and molecular methods. For morphological characterization, colony characteristics on malt extract agar and microscopic appearance of methyl blue stained smear, under 40x and oil immersion (100x were observed. For biochemical characterization, urea hydrolysis, sugar fermentation (MOF – Microbial Oxidation Fermentation test), fatty acid hydrolysis, nitrate assimilation, starch like substance production, citric acid production, Diazonium Blue B reaction (DBB) and growth at 37° C were performed (Barnett *et al*., 2000). Finally, species identification of the isolates was performed by sequencing of ITS region as per Harju *et al*. (2004) with ITS primers (Forward ITS 1: 5' -TCC GTA GGT GAA CCT GCG G- 3' and Reverse ITS 4 - 5' -TCC TCC GCT TAT TGA TAT GC- 3') (White *et al*., 1990). The amplified fragments of approximately 580 bp containing the ITS 1, 5.8 S and ITS 2 regions were used for the sequence similarity search using NCBI BLAST.

2.3. Effect of physico-chemical parameters for extracellular enzyme production

Different yeast isolates showing highest activity on plates for each of the four enzymes were selected for optimizing growth conditions. PON5-7, CTV3-1, KDI4-17 and KDI5-8 were the strain numbers given for the isolates studied for optimization of growth for the maximum production of protease, amylase, lipase and ligninase, respectively.

Various growth parameters like pH, salinity, substrate concentration and temperature were optimized for the production of enzymes under study (Kutty *et al*., 2012). Selected yeast strains were inoculated into malt extract broth, grown at 28 ± 2º C for 48 hours and the optical density of the culture suspension was taken at 540 nm with the help of a UV-VIS spectrophotometer. Later, the OD was adjusted to 1 by dilution with sterile water and 10µl of this cell suspension was used as inoculum. The experiment was performed in triplicates and incubated at 28 ± 2º C for 5 days except for temperature optimization. The optical density/absorbance was measured using UV-VIS spectrophotometer at 540 nm and was used for further calculations. The mean absorbance value of the triplicate samples and their standard deviation were calculated.

Malt extract broth supplemented with casein, starch and tributyrin were used for the production of protease, amylase and lipase, respectively. Crawford's broth supplemented with tannic acid was used for testing ligninase production.

pH: Malt extract broth with respective substrates for protease, amylase, lipase and ligninase were prepared at pH 5, 6, 7, 8 and 9 in triplicates, inoculated with selected strains and incubated as mentioned.

Salinity: Malt extract broth with respective substrates for protease, amylase, lipase and ligninase of different salinities 5ppt, 10 ppt, 15 ppt, and 20 ppt at optimized pH were prepared in triplicates, inoculated with selected strain and incubated as mentioned.

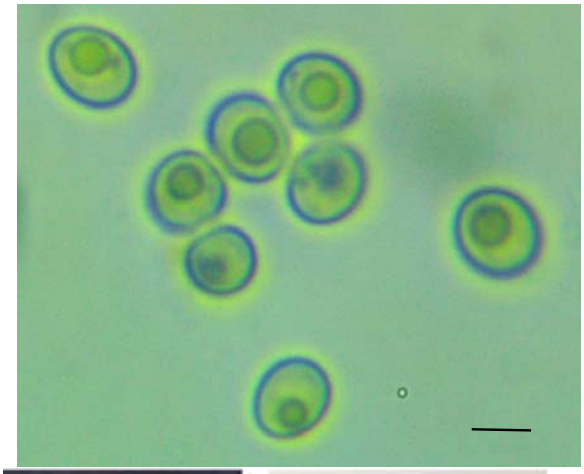
Substrate concentration: Malt extract broth with respective substrates for protease, amylase, lipase and ligninase at optimized pH and salinity and varying substrate concentrations viz 0.5%, 1%, 1.5%, 2%, 2.5% for protease, 0.25%, 0.5%, 1%, 1.5%, 2% for amylase and lipase, 0.1%. 0.25%, 0.5%, 0.75%, 1% for ligninase was prepared in triplicates, inoculated with selected strain and incubated as mentioned.

Temperature: Malt extract broth with respective substrates for protease, amylase, lipase and ligninase at optimized pH, salinity and substrate concentrations were prepared in triplicates, inoculated and incubated at temperatures 20º C, 25º C, 30º C, 35º C and 40º C.

3. Results

3.1. Screening for enzyme activity and Identification of potent isolates

A total of 486 yeast isolates from the mangrove sediments were screened for the presence of different extracellular enzymes, out of which 429 isolates showed hydrolytic activities for one or more enzymes under study. Plates with medium containing respective substrates showing hydrolytic activities for the enzymes under study were shown as Figure 1. Clearance zone was formed by precipitation after the addition of 1M HCl for protease while clearance zone was formed after flooding with Gram's iodine for amylase. Lipase and ligninase activity were determined by the formation of clearance and halo/brown zones respectively. The isolates which showed maximum activity at 72 hours of incubation as measured by the increase in zone of clearance every 24 hours were selected and identified. PON5-7, CTV3-1, KDI4-17 and KDI5-8 were the potent isolates selected for protease, amylase, lipase and ligninase activity, respectively. The colony characteristics of the isolates on malt extract agar showed mucoid and glossy appearance with irregular margins. Microscopic examination showed oval shaped, hyphated / pseudohyphated cells which asexually reproduced by budding (Fig. 2). The biochemical characteristics of the 4 strains were studied (Table 1). The amplification and sequencing of the ITS region of the yeast DNA confirmed that all the selected strains belong to *Candida tropicalis* when compared with the NCBI GenBank database, with 100% sequence homology. The GenBank accession numbers obtained were MW 617308, MW 617310, MT 149215 and MW 617305 for isolates PON5-7, CTV3-1, KDI4-17 and KDI5-8, respectively.



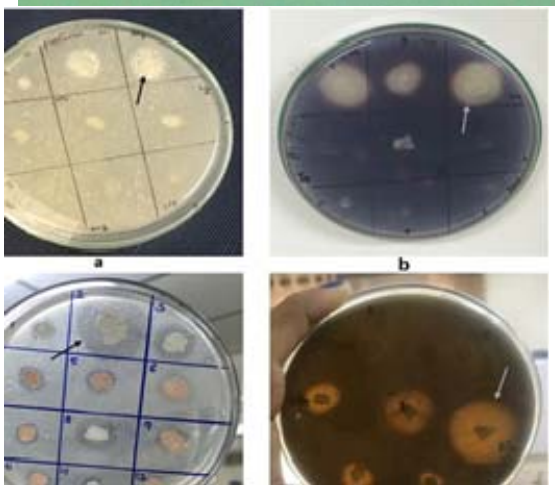


Figure 1: Plates showing hydrolytic activities of enzymes. Arrows indicate the clearance / halo zones formed due to the hydrolysis of substrates. **a)** Protease – clearance zone formed by precipitation after the addition of 1M HCl. **b)** Amylase – clearance zone after Gram's iodine treatment **c)** Lipase – clearance/ halo zone. **d)** Ligninase – formation of halo zone

Figure 2: Microscopic appearance of methylene blue stained *C. tropicalis* under 100x (oil immersion) magnification.

Table 1: Biochemical characterization of different *C. tropicalis* isolates in the present study

Sl. No.	Tests	Results			
		PON5-7	CTV3-1	KDI4-17	KDI5-8
	Urea hydrolysis	-	-	-	-
	Glucose fermentation (MOF test)	+	+	+	+
	Fatty acid hydrolysis	+	+	+	+
	Nitrate assimilation	-	-	-	-
	Starch like substance production	-	+	-	+
	Citric acid production	-	-	-	+
	Diazonium Blue B reaction (DBB)	-	-	-	-
	Growth at 37° C	+	+	+	+

3.2. Study of growth parameters for extracellular enzyme production

Study of various parameters for efficient growth and maximum biomass yield which result in maximum extracellular enzyme production was determined for the enzymes protease, amylase, lipase and ligninase. The growth/biomass in the culture media was measured on the basis of turbidity obtained by measuring the absorbance at 540nm wavelength. Growth parameters of isolates PON5-7, CTV3-1, KDI4-17 and KDI5-8 (all identified as *C. tropicalis*) were studied for the maximal production of its protease, amylase, lipase and ligninase enzymes, respectively.

*3.2.1. **Effect of pH:** The effect of the pH on the growth of yeast strains under study is graphically summarized as Fig. 3.*

Protease: Maximum growth / absorbance at 540 nm was observed at pH 8, when the pH of the media was pre-adjusted from 5-9. At acidic pH, the growth was very low but increased as the media became basic. The optimum pH 8 was maintained for the culture media for further procedures.

Amylase: Maximum growth / absorbance at 540 nm was observed at acidic media of pH 5 and the growth decreased as the media became basic. The optimum pH 5 was maintained for the culture media for further procedures.

Lipase: The isolate showed increased growth / maximum absorbance at 540 nm as the pH of the media became basic and maximum growth was observed in media with pH 9. The optimum pH 9 was maintained for the culture media for further procedures.

Ligninase: Maximum growth/ absorbance at 540 nm was observed in basic media with pH 9 and no much significant growth was seen in acidic media. The optimum pH 9 was maintained for the culture media for further procedures.

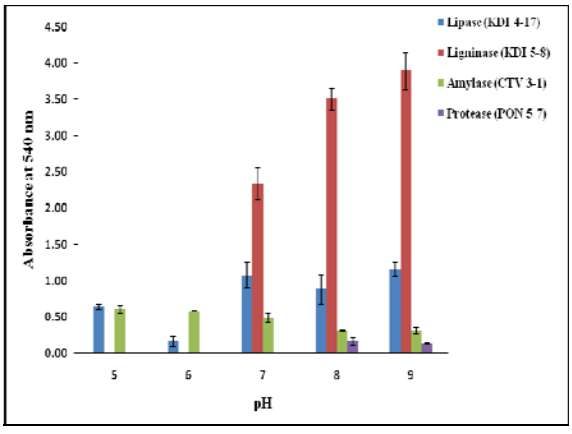


Figure 3: Effect of different pH in media on the growth and enzyme production potential of selected strains of *C. tropicalis* in the present study

*3.2.2. **Effect of salinity:** The effect of the salinity of media on the growth of yeast strains under study is graphically summarized as Figure 4.*

Protease: Maximum growth / absorbance at 540 nm was observed when the salinity of the media was adjusted to 5 ppt and decreased as the salinity increased. The optimum salinity of 5 ppt was maintained in the culture for further procedures.

Amylase: The culture showed maximum growth/ absorbance at 540 nm in media with salinity of 5 ppt and gradually decreased as the salinity increased. The optimum salinity of 5 ppt was maintained in the culture for further procedures.

Lipase: Maximum growth/ absorbance at 540 nm was observed in media with salinity of 10 ppt and reduced at other concentrations. The optimum salinity of 10 ppt was maintained in the culture for further procedures.

Ligninase: The media with salinity of 5 ppt showed maximum growth/ absorbance at 540 nm and it decreased in higher salinities. The optimum salinity of 5 ppt was maintained in the culture for further procedures.

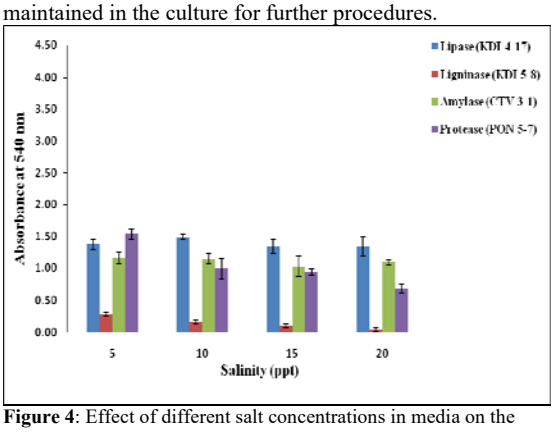


Figure 4: Effect of different salt concentrations in media on the growth and enzyme production potential of selected strains of *C. tropicalis* in the present study

*3.2.3. **Effect of substrate concentration:** The effect of the salinity of media on the growth of yeast strains under study is graphically summarized as Figure 5.*

Protease: The media with substrate concentration of 2% casein showed maximum growth/ absorbance at 540 nm while it decreased in lower and higher substrate concentrations. The optimum substrate concentration of 2% casein was maintained in the culture for further procedures.

Amylase: Maximum growth / absorbance at 540 nm was observed in media with substrate concentration 1% starch and the growth decreased at lower and higher concentrations. The optimum substrate concentration of 1% starch was maintained in the culture for further procedures.

Lipase: The media with substrate concentration of 1% tributyrin showed maximum growth/ absorbance at 540 nm while it decreased in lower and higher substrate concentrations. The optimum substrate concentration of 1% tributyrin was maintained in the culture for further procedures.

Ligninase: Maximum growth/ absorbance at 540 nm was observed in media with substrate concentration 0.5% tannic acid and the growth decreased at lower and higher concentrations. The optimum substrate concentration of 0.5% tannic acid was maintained in the culture for further procedures.

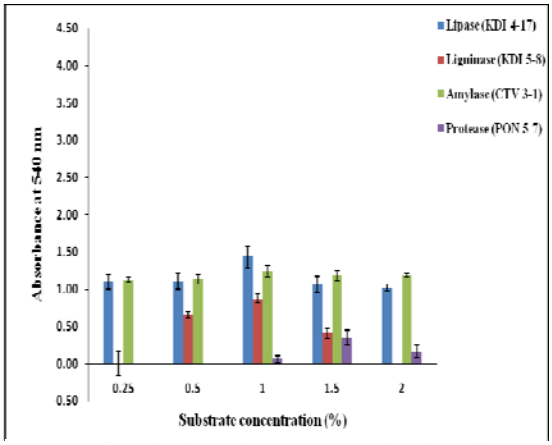


Figure 5. Effect of various substrate concentrations in media on the growth and enzyme production potential of selected strains of *C. tropicalis* in the present study

3.2.4. ***Effect of temperature:*** *The effect of various incubation temperatures on the growth of yeast isolates cultured under optimized media conditions under study is graphically summarized as Figure 6.*

Protease: Maximum growth / absorbance at 540 nm was obtained when the culture was incubated at 40°C and below that temperature there was reduction in growth of the culture

Amylase: Maximum growth / absorbance at 540 nm was obtained when the culture was incubated at 35°C but above and below that temperature the growth showed a decreasing pattern.

Lipase: Maximum growth / absorbance at 540 nm was obtained when the culture was incubated at 35°C but above and below that temperature the growth showed a decreasing pattern.

Ligninase: Maximum growth / absorbance at 540 nm was obtained when the culture was incubated at 40°C and below that temperature there was reduction in growth of the culture.

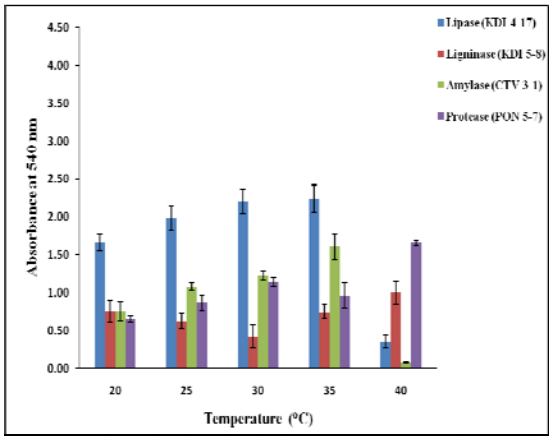


Figure 6. Effect of various incubation temperatures on the growth and enzyme production potential of selected strains of *C. tropicalis* in the present study

4. Discussion

In the present study, the effects of various growth parameters including pH and salinity of the media, substrate concentration and incubation temperature were determined to obtain maximum growth and enzyme production from selected yeast strains. Though many of the yeast isolates showed significant extra cellular enzyme activities, those potent strains which showed maximum activity for particular enzymes as determined by the measurement of clearance zones on enzyme plates with respective substrates were screened and studied. All the four isolates selected for the study of enzymes protease, amylase, lipase and ligninase were identified as *C. tropicalis*. *C. tropicalis* is considered as a biological and biotechnological important yeast strain due to its applications in agricultural, fermentation and chemical industries (Kuiran *et al*., 2010). Still, there are less studies conducted on the hydrolytic enzyme potential of *Candida* species, especially *C. tropicalis* isolated from marine and mangrove samples.

The phenotypic characteristics expressed by microorganisms are greatly influenced by the environment they exist in, and it acts as their adaptation strategy. Yeasts, like *C. tropicalis* that can produce extracellular enzymes, have specific genes to express particular enzymes (Yan *et al*., 2005). The ability of these genes to express enzymes in turn are impacted by their environment as well as the nutrients present there (Amadi *et al*., 2020). Hence, understanding and proper optimization of the factors affecting the growth of yeast to achieve maximum biomass and enzyme production in culture is necessary.

C. tropicalis has a wide distribution and has been isolated from different marine habitats including mangrove ecosystem (Kuiran *et al*., 2010). A study conducted in marine oil degrading yeast strain *C. tropicalis* SD 302 shows maximum growth at pH 7, 15 ppt salinity and 30°C temperature (Kutty *et al*., 2012). The optimum culture conditions for maximum biomass yield and increased production of protease from *C. tropicalis* in our study was found to be pH 8, 5 ppt salinity, 2% casein as substrate concentration at 40°C temperature. This reveals the potency of the isolated strain in present study compared to the marine counterpart, due to its maximum production at high pH, high temperature and medium salinity. Studies on genus other than *Candida* have also shown that the optimum culture conditions for the high productivity of yeast protease obtained *Metschnikovia pulcherrima* and *Wickerhamomyces anomalus* was at basic pH at temperature ranging from 40-45° C, with salinity between 5-10ppt (Schlander *et al*., 2017). Research on yeast amylases showed that higher enzyme production was seen normally at acidic pH ranging from 5-6 at 1% substrate concentration with 5-10 ppt salt concentrations at temperature ranging from 30-35° C (Nahas and Waldemarin, 2002; Souza and Magalhaes, 2010). In our study, the optimum culture conditions for large scale production of yeast amylase from C.*tropicalis* were found to be at an acidic pH of 5, with 5% salt concentration and 1% starch as substrate at incubation temperature of 35° C. Lipases hydrolyze acylglycerides and are highly valuable enzymes in detergent industry and also in biodegradation of oil residues (Hasan *et al*., 2006). Recently yeast lipases have been widely used in developing novel techniques like biosensing, organic synthesis of drugs and synthesis of optically active compounds in pesticide industries (Vakhlu and Kour, 2006). Previous studies on yeast lipases mainly, those from *C. tropicalis*, showed that the optimal pH, temperature and salinity for their production were between

6.0-8.5, 35-40º C, 5-10 ppt, respectively at 1% substrate concentration (Abu *et al*., 2017; Alamia *et al*., 2017). The culture conditions for the production of lipases from *C. tropicalis* in our study were optimized as pH 9, 10 ppt salinity, 1% tributyrin as substrate at an incubation temperature of 35°C. Though yeast ligninase have not been studied much, interest has been recently increased due to its applications in wood, paper and cosmetic industries (Malgas *et al*., 2017). Optimization of the culture conditions required for the high yield and enzyme production of ligninase producing yeasts would be of great help in the development of its fermentation process. In our study, the optimum culture conditions for the large scale production of ligninase from *C. tropicalis* was found to be at pH 9, 5 ppt salinity, with 2% tannic acid as substrate at an incubation temperature of 40°C.

During the large-scale fermentation of enzymes, especially in detergent and chemical industries, the alkalinity and temperature of the culture media tend to increase as the procedure progresses. So, it is advisable to use strains which can survive, grow and produce enzymes at higher temperatures, increased pH and saline conditions (Gurung *et al*., 2013; Arnau *et al*., 2019). Since mangrove sediments experience extreme environmental alterations, the microbes including yeasts isolated from it will be adapted to such variations and they use this property to survive in different cultural conditions. Also, the biomolecules like extracellular enzymes produced by them would be able to withstand such extreme parameters (Thatoi *et al*., 2013; Capdeville *et al*., 2019). This helps in performing hassle free scaling up process for the mass production of these biomolecules from yeasts. The optimum culture conditions needed by yeast *C. tropicalis* for the maximum biomass and enzyme production in the present study were in accordance with the requirements for the large scale production of enzymes. Hence, *C. tropicalis* can be exploited as a suitable candidate for the industrial and biotechnological production of hydrolytic enzymes.

5. Conclusion

The quest for novel and improved microbial strains that can produce industrially important enzymes is a continuous process. Since, yeasts are one of the most active extracellular enzyme producers and can be fermented using cheap substrates; they have gained massive attention during recent years. We have studied the effect of various culture parameters including pH, substrate concentration, salinity and incubation temperature on the growth of selected strains of *C. tropicalis* for the production of hydrolytic enzymes protease, amylase, lipase and ligninase. The results showed that the optimum growth conditions required for this strain would be favorable in the large- scale fermentation process for producing extracellular enzymes. Hence, the present study suggests that the yeast *C. tropicalis* isolated from mangrove sediments effectively produce extracellular hydrolytic enzymes and can be utilized for various biotechnological and industrial applications.

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