PRODUCTION OF POLYSACCHARIDE DEGRADING ENZYMES BY YEASTS.

Jhilam Majumder and Dr. Rina Rani Ray*

**Associate Proffesor, PG Dept. of Zoology, Bethune College*

Abstract

Four yeast strains, namely *Saccharomyces cerevisiae,Candida albicans, Pichia stipitis* and *Debaryomyces hansenii* were cultivated in presence of various carbon sources including starch, cellulose, hemicelllulose, pectin and chitin.The cellulosic and hemicellulosic residues followed by starchy substrates were found to be preferred substrates for all the four strains.Amongst the strains, *Saccharomyces cerevisiae* showed the most rapid growth followed by *Candida albicans.* Moderate growth was shown by *Pichia stipitis,* while *Debaryomyces hansenii* showed poor growth. All the strains were found to produce extracellular hydrolytic enzymes like amylase. Cellulase, avicelase , xylanase, pectinase and chitinase. Although the productive efficacy was highest in *Saccharomyces cerevisiae* , other non-saccharomyces yeasts were also found to synthesize the enzymes. Amongst the four yeast strains tested, *Pichia stipitus* and *Candida albicans* were found to be more resistant to antifungal compounds than the other two.

Introduction

The most abundant renewable form of biomass is lignocellulose comprising between 50 and 90% of all plant matter which is composed of three major components, cellulose, hemicellulose, and lignin (Kricka *et al*, 2014). On the other hand, starch constitutes the most abundant rapidly renewable source of energy for living organisms (Hostinova, 2002). Pectin is a structural heteropolysaccharide contained in the primary cell walls of terrestrial plants, whereas chitin is a long-chain polymer of a *N*-acetylglucosamine, a derivative of glucose, and is found as characteristic component of the cell walls of fungi and the exoskeletons of arthropods and mollusks.

These polysaccharides are degraded by respective hydrolytic enzymes. Starch is hydrolyzed to glucose units by amylase (E.C.3.2.1.2), cellulose and avicel is degraded by endoglucanase (EC 3.2.1.4) and avicelase (EC 3.2.1 91) respectively, xylan or hemicelluloses is degraded by xylanase (E.C.3.2.1.8), pectin is cleaved by polygalacturonase (EC 3.2.1.15) and chitin is degraded by chitinase (EC 3.2.1.14) .

Yeasts are unicellular fungi which are known for their ability to ferment sugar for the production of ethanol with the help of different hydrolytic enzymes (Vazquez *et al*, 2004). Different yeasts are able to utilize different carbon sources, and nutritional selectivity determines yeast species diversity in particular niches. Although Saccharomyces is widely used for its amylolytic and pectinolytic activities, the potential of hydrolytic enzyme production by non- saccharomyces yeasts are yet to be explored.

The present study deals with the preliminary investigation of on saccharifying enzyme synthesis by four ethanolic yeast strains.

Materials and methods

Microorganism: Four Yeast strains namely *Saccharomyces cerevisiae*, *Pichia stipitis*, *Candida albicans*, *Debaryomyces hansenii* were collected from **National Collection of Industrial Microorganisms** (NCIM), Pune .These were maintained in Martins Rose Bengal agar Medium (33.6 gm /liter) and were incubated at 28°C for 48 hours.

Morphological characterization: The strains were stained by cotton blue and seen under light microscope. The appeared colonies were checked for their colour, outline and other features.

***In situ* detection of amylase, cellulase, xylanase , pectinase , avicelase and chitinase activity:**

The strains were grown on a solid basal medium (BM) composed of (gl^{-1}): peptone 0.9; $(NH_4)_2HPO_4$ 0.4; KCl 0.1; $MgSO_4.7H_2O$ 0.1 and agar 20 (pH 7.0) for 48-72hours at 28°C (Chatterjee et al, 2011). The culture plates were supplemented individually with starch, carboxy methyl cellulose, xylan, pectin, avicel and chitin as sole carbon source. The ability of the strains to grow on these plates revealed their efficacy to produce the respective key enzymes namely amylase, cellulase, xylanase pectinase , avicelase and chitinase.

For *in situ* detection of amylase activity, the starch agar plates with yeast colonies were flooded with iodine solution (Ray, 2001) whereas cellulose agar and xylan agar plates with yeast colonies (Gohel *et al,* 2014) were irrigated with 0.2% congo red solution. The pectin agar plates were irrigated with iodine-potassium iodide solution (1.0g iodine, 5.0g potassium iodide and 330ml H_2O) to detect clearance zones. The halo formed around each yeast colony indicated the production of respective extracellular enzymes by the particular strain.

Enzyme extraction and assay: The yeast strains were cultivated in 100-ml Erlenmeyer flasks, each containing 10 ml of agar less basal medium (BM) separately supplemented with different carbon sources (as mentioned above) for 24-96 days at 28°C. The grown culture was centrifuged at 10,000 rpm for 10 minutes and the supernatant was used as the crude enzyme. The enzyme activity was detected by incubating the assay mixture (1 ml) containing equal volumes of properly diluted enzyme and 0.5-1% (w/v) substrate in 0.1 (M) phosphate buffer (pH 7) at 50° C for 10 min. The reducing sugar produced was measured spectrophotometrically at 540 nm
(Shimadzu, Japan) following the dinitrosalicylic acid method (Bernfeld, 1955). The substrates used were starch (1% w/v), carboxy methyl cellulose (1% w/v), xylan (0.5% w/v), avicel (0.5%), (Mukherjee *et al*, 2011), chitin (0.1%) and pectin (0.5% w/v) for estimation of amylase, cellulase, xylanase , avicelase, chitinase and pectinase respectively. A standard curve of glucose was prepared to calculate the concenration of amylase , cellulase and avicelase, whereas xylose , N -acetyl-D-glucosamine and galacturonic acid were taken as the standards for estimating xylanase and pectinase activity respectively. The heat killed or inactivated enzymes were used as respective controls (Saha and Ray, 2015).

Effect of antifungal on growth of the yeast strains: Antifungal susceptibility testing was carried out by the modified Kirby-Bauer method. Small filter papers soaked with antifungal solutions were placed onto a culture plate of growing yeast. Appearance of a clear ring or zone of inhibition around the yeast colony indicated the susceptibility of the strain against that particular antifungal compound.
Each experiment was replicated thrice and their values were averaged.

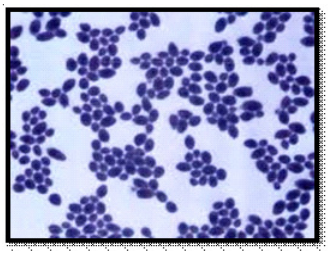
Results and discussion

The four yeast strains stained with crystal violet showed distinct morphological features (Fig 1), of which *S. cerevisiae* cells were round to ovoid, 5–10 ì m in diameter with unicellular, ellipsoid cell buds. *Candida albicans* was a diploid fungus that was found to grow both as yeast and filamentous cells.
The colonies of the yeast strains (Fig 2) revealed that on dextrose-agar plate, most rapid growth was shown *by Saccharomyces cerevisiae followed* by *Candida albicans.* Moderate growth was shown by *Pichia stipitis,* while *Debaryomyces hansenii* showed poor growth.
Although starch was proved to be the most preferred carbon source for all the working strains of yeasts, these strains took 72-96 hours to ferment the starch substrate, whereas hemicellulosic substrate (xylan) was effectively hydrolyzed within 24-48 hours of cultivation. On the other hand, pectin and chitin were found to take a longer fermentation time (Fig 3).
Amongst the four yeast strains tested, *Saccharomyces cerevisiae* was found to be the best producer of all extracellular enzymes, followed by *Candida albicans*, whereas *Debaryomyces hansenii* did not show significant enzyme synthesizing efficacy (Fig 4).
These strains showed a higher affinity towards cellulosic and hemicellulosic residues followed by starchy substrates. Growth was not much encouraged in presence of pectin. On the other hand, *Pichia* was found to be a good producer of pectinase (Moharib *et al,* 2000)
Different strains of yeasts showed various susceptibility patterns against different antifungal compounds (Table 1). Highest resistance was showed against cycloheximide , a compound that blocks translocation step in protein synthesis, whereas except *Pichia*, all other yeast strains tested were found to be susceptible in presence of Cotrimazole, that alters the permeability of the fungal cell wall and thereby destroys the cell. Amongst the four yeast strains tested, *Pichia stipitus* and *Candida albicans* were found to be more resistant to antifungal compounds than the other two.

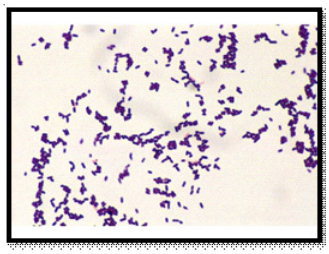
Table 1. Effect of antifungal compounds on the growth of yeast strains.

Antifungals	***S.cerevisae***	***P.stipitus***	***D.hansenni***	***C.albicans***
Cotrimazole	-	+	-	-
Cycloheximide	-	+	+	+
Flucanazole	-	+	-	+
Griseofulvin	+	-	-	+

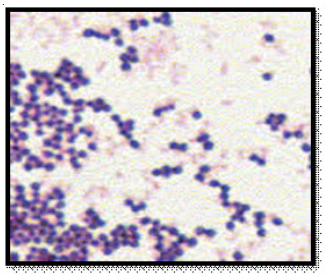
Fig 1. Micrographs of the yeast strains
A: *Saccharomyces cerevisiae*, **B** : *Pichia stipitis*,
C: *Candida albicans*, **D** : *Debaryomyces hansenii.*



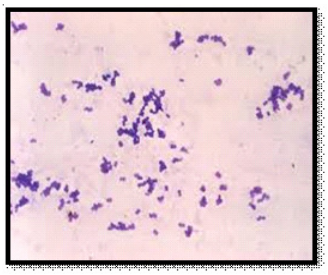
A



B



C



D

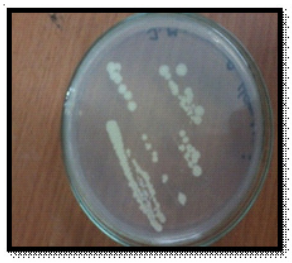


Fig 2.
Colonies of the yeast strains on glucose agar plates
A: *Saccharomyces cerevisiae*, **B** : *Pichia stipitis*,
C: *Candida albicans*, **D** : *Debaryomyces hansenii.*

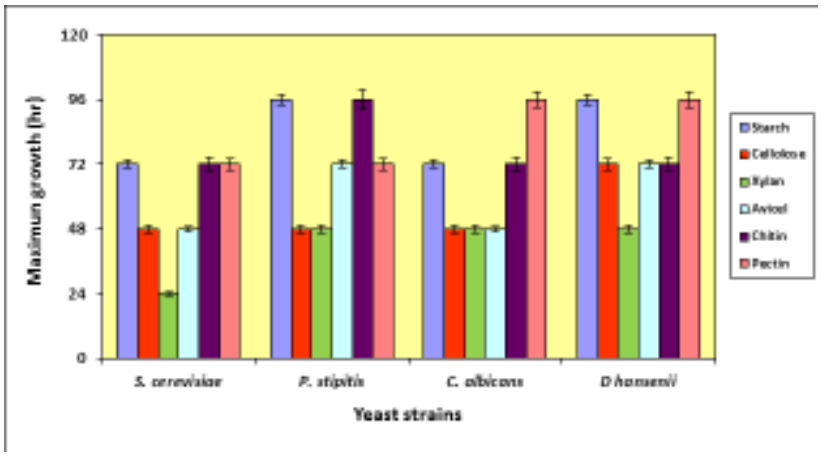


Fig. 3. Relative growth of the yeast strains in various carbon sources.

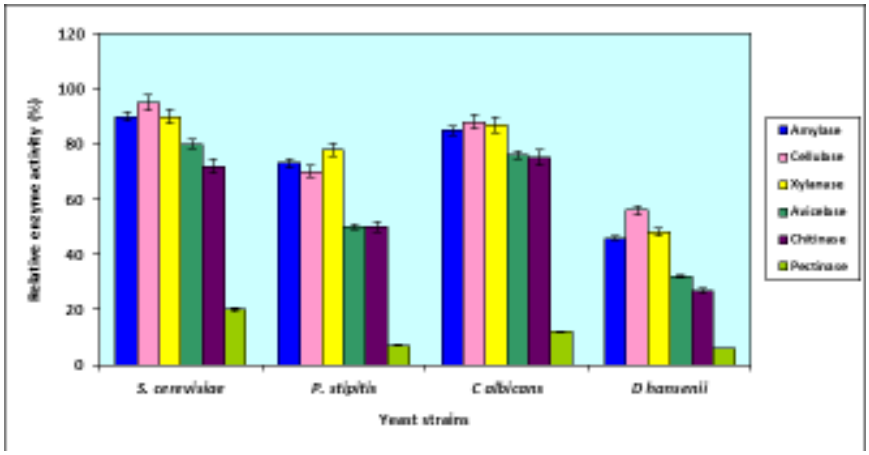


Fig 4 Relative production of extra cellular enzymes by different yeast strains.

Conclusion

Enzymatic activity in yeasts is of interest in connection with the use of the yeasts or the enzymes produced by them to enhance the varietal character of the wine (Orliæ *et al*, 2007). Among the desirable enzymes in a wine yeasts are the amylase, hemicellulase, cellulases (endo and exoglucanase) and pectinase. Although a range of non saccharomyces yeasts namely Candida, Debaryomyces, Pichia were found to produce a wide range of useful extracellular enzymes during the initial phase of wine fermentation (Strauss *et al,* 2001), the present study confirmed the fact showing almost similar production efficacy of Candida albicans in comparison to *Saccharomyces.*

Reference

Bernfeld, P. (1955) .Method. Enzymol. 1: 149–150.

Chatterjee , S., Ghosh, B and Ray, R. R. (2011). Isolation and characterization of local yeast strains from waste fruit juices, jaggery and dahi samples. Int. J. Chem. Sci.: 9(2) :647-656.

Gohel,H.R., Contractor, C.N., Ghosh, S.K. (2014).Vincent J. Braganza A comparative study of various staining techniques for determination of extra cellular cellulase activity on Carboxy Methyl Cellulose (CMC) agar plates. Int.J.Curr.Microbiol.App.Sci. 3(5): 261-266

Hostinova, E (2002) Amylolytic enzymes produced by the yeast Saccharomycopsis _buligera. Biologia, Bratislava, 57/ Suppl. 11: 247-251

Kricka ,W., Fitzpatrick, J and Bond U (2014). Metabolic engineering of yeasts by heterologous enzyme production for degradation of cellulose and hemicellulose from biomass: a perspective Front. Microbiol. 5:174. doi: 10.3389/ fmicb.2014.00174

Moharib SA, el-Sayed ST and Jwanny EW. (2000) Evaluation of enzymes produced from yeast. Nahrung .44(1):47-51.

Mukherjee S, Karmakar M, Ray RR (2011) Production of extra cellular exo glucanase by *Rhizopus oryzae* from submerged fermentation of agro wastes. Rec. Res. Sci. Technol. 3(3): 69-75.

Orliæ, S., Katarina, H; Urliæ, B., Red•epoviæ, S. (2007). Screening for the production of extracellular hydrolytic enzymes by *Saccharomyces* wine yeasts isolated from Croatian wine yards .Periodicum biologorum 109,: 201-204

Ray, R. R. (2000). Purification and characterization of extracellular â-amylase of *Bacillus megaterium* $B_{6.}$ *Acta Microbiologia et Immunologica Hungarica*. 47(1):29-40

Saha , P and Ray, R. R. (2015). Production of Polysaccharide degrading enzymes by the gut microbiota of *Leucinodes orbonalis* and *Bactrocera dorsalis.* J. Entomol.Zool. Studies. 3 (1): 122-125

Strauss, M.L., Jolly, N.P., Lambrechts, M.G. and van Rensburg, P. (2001) Screening for the production of extracellular hydrolytic enzymes by non-Saccharomyces wine yeasts. J Appl Microbiol. 9(1):182-90.

Vazquez, F., Herrera, M.D.V., de Figueroa, L.I.C and Toro, M.E. (2004) Extracellular Hydrolytic Enzymes Produced by Yeasts. Methods . Biotechnol. 16: 283-297