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Characterization of Yeast isolates NB1 and NB3 from ITS Region of the Leaf of Alstonia Scholaris

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ABSTRACT

For experimental study , refrigeration stored stock culture of two isolated novel yeast strains namely NB1 and NB3 were used . These isolates were freshly grown at pH 5.8 and 30°C temperature for culture revival. For the characterization of isolated yeast strains; genomic DNA isolation and identification of strains using ITS primer ITS 1 and ITS 4 through PCR reaction was conducted. Growth curve analysis of isolated (NB1 and NB3) yeast strain shown that both the isolated yeast strains (NB1 and NB3) revealed varying growth pattern, both of them were faster growing strains with short life span. For phenotypic characterization: growth parameters, morphological and physiochemical characterization was carried out as Hydrogen peroxide test, Thermo tolerance test, Glycerol test, Galactose test , Growth at different temperature , Methyl Methane Sulphonate and pH of the isolated yeast strains , was performed. All these tests revealed that the isolated yeast strains were highly resistant at different pH conditions on YPD (Yeast Peptone Dextrose) agar medium (pH= 5.8,7,8), at higher concentration of H_2O_2, Glycerol, Galactose reveals tolerance but in case of MMS drug concentration shows little tolerance when spotted with control BY474 1 Reference laboratory strain on YPD agar plates with incubation at 30°C for 48 hours.

Introduction

Yeast have a great potential application primarily in brewing , wine making , baking, distilled spirits production ,also in biomass production. Saccharomyces cerevisiae (baker's yeast) is promisingly used in research to increase the yield of the production of bio-ethanol from sugars through zymase, cellulases and xylanases enzymes. Saccharomyces have been genetically engineered to ferment xylose, one of the major fermentable sugars present in cellulosic biomasses, such as agricultural residues , paper waste s, and wood chips. Such a development contributes that ethanol can be efficiently produced from more inexpensive feed stocks, making cellulosic ethanol fuel a more competitively priced alternative to gasoline fuels. Many yeast strains can function both under anaerobic as well as aerobic conditions of environment, switching their metabolism types easily (Otterstedt K *et al* ., 2004). Both classical approaches as well as modern strategies of gene manipulations are applied to generate variants relevant to work under industrial specific conditions (Steensels J *et al*., 2014).

In yeast processing factories the main raw materials used are beet or cane molasses , due to their high sucrose content and low waste cost, yeast are also excellent source of single cell protein (SCP), most popularly used species are candida , Hansenula , Pichia, Torulopsis , and Saccharomyces (P J, Sarlin & Philip, Rosamma. 2016). but marine yeast is also valuable source of high protein .

For pharmaceutical industry, lipases are used to resolve racemic mixtures of alcohols or carboxylic acids through asymmetric hydrolysis of acyl derivatives. Candida antarica Lipase , isoform B (CAL-B) Commercial CAL-B is available either in free, lyophilized , and immobilized forms (onto Lewatit VP OC1600 Poly (methyl methacrylate – co-divinylbenzyene)- Novozyme435,(Chirazyme L2-C2). The immobilization of CAL-B onto different supports may result in different activity and enantioselectivity and may be tool of control of selectivity of the hydrolysis. This feature was used for the resolution of racemic mixture of 2-o-butyryl – 2 phenylacetic acid- precursor of both enantiomers of mandelic acid and for the enantioselective hydrolysis of 3- phenylglutaric dimetyl diester- precursor in the drug synthesis (e.g., HIV inhibitor) (Hirose Y *et al*;1992) . Lipases enzymes are applied in various industries for either of lipid modifications and synthesis of special compounds: pharmaceuticals, polymers, biodiesels, and biosurfactants(Weber et al; 2011). The most important application of lipases in organic synthesis is esterification important for the resolution of racemic mixtures of secondary alcohols and carboxylic acids. Chiral secondary alcohols serve as intermediates for pharmaceutical synthesis (Margolin al *et al*;1993). Another enzyme invertase possess industrial usage in production of invert syrup equimolar mixture of fructose and glucose, released from sucrose, which is applied in food and beverage industries. Commercially invertase is produced mainly by Saccharomyces cerevisiae (Baker's Yeast) or Saccharomyces carlsbergensis. Invertase is also suited for the manufacture of artificial honey , plasticizing agents for cosmetics , pharmaceutical and paper industries , and enzyme electrodes for the detection of sucrose (Seebach D *et al*;1985). Additionally , it can be applied for the synthesis of probiotic oligosaccharides like non- digestible oligosaccharides (NDO) e.g lacto sucrose (Williams RE and Bruce NC, 2000).

Materials and Methods

Material required – Epppendorf,'s , eppendorf's stand , 70 % ethanol, 100% Chilled ethanol, Ice , Autoclaved water, Petri plates , Beaker, centrifuge , vortex, Laminar air flow, Incubator etc.

Reagents required - : (Breaking buffer)

Reagents	Stock Concentration	Working concentration
Triton X	4%	2%
SDS	10%	1%
Nacl	1M	100Mm
Tris HCL	1M	10Mm
EDTA	0.5 M	1Mm
Sodium Acetate	1M	500Mm

Morphological Characterization

According to the method of Kurtzman *et al* ;2011 morphology of the vegetative cells of yeast was observed grown in Liquid and Solid media.

Fig1 :- Colonies of NB3 and NB1 Strains on YPD agar plates after incubation for 48 hrs at 30 °C

Genomic DNA Isolation from isolated yeast strain

Initally, yeast culture is inoculated in 10ml of YPD broth and is incubated at 30°C for overnight. On next day centrifugation is carried out at 3000rpm at 4°C for 5 minutes . Then supernatant was discarded, the pellet was taken and dissolved in 200 µl breaking buffer , 200 µl phenol, also in 200 µl of SDS.The suspension was vortexed for 1 minutes and then placed on ice for 1 min , repititon of this step once again. After that, addition of 1 Molar of tris (200 µl), vortexed for 1 minute, this suspension is placed on ice for 1 min. Then Centrifugation at 15000rpm was performed for 10 min at 4°C . Subsequently, ,aqueous layer was pipette out into another eppendorf tube and 1ml of 100 % of chilled ethanol was added into it. Again , centrifugation was performed at 15000rpm at room temperature for 10 minutes. Then the supernatant was discarded and pellet was taken ,1 M Tris (100 µl) , RNAase (3 µl). was added, placed at 37°C for 20 min . Also, 40 m sodium acetate (40 µl) was added. Later 1 ml of chilled ethanol was added and placed on ice for 20 min. Then centrifugation at 15000 was done at room temperature for 10 min, Pellet was taken and 70 % of 1 ml ethanol was added for washing. Pellet was air dried and suspended in 10mM tris (20 µl) incubation was performed at 30°C for half an hour for dissolving pellet completely.

Gel Electrophoresis - For 50ml of 1% gel. 0.5 g of agarose was weighed and dissolved in 50 ml of 1X TBE buffer by heating till the solution become transparent .The mixture was then cooled for 10 min, subsequently 3 µl EtBr (Ethidium Bromide) was added. The comb was placed over the gel casting tray the mixture was poured into it, then the gel it is allowed to solidify at room temperature for 15 min to form wells. After solidification of the gel, the samples were loaded into the wells. Later on this whole electrophoretic apparatus with the samples is allowed to run under the application of 100V power supply for almost ½ hour. After that the gel is removed from the apparatus and visualize under the U.V transilluminator for visible isolated DNA sample bands .Also, in case of RNA contamination within the samples, the samples were given 3 µl of RNAase treatment to remove RNA from the samples.

Note:- 3 µl DNA sample and 2 µl of bromophenol dye (tracking dye) is mixed together and loaded into the wells. Then the samples DNA bands were examined under the U.V transilluminator to visualize isolated DNA bands.

Polymerase chain Reaction [PCR]amplification of isolated yeast strains

The DNA amplification process was performed in polymerase chain reaction (PCR) tubes, each containing a 100µL reaction mixture. The composition of the reaction mixture includes 2µL of template DNA (at a concentration of 50 ng/µL), 10 µL of 1X PCR buffer with $MgCl2$, 8 µL of each dNTP (dTTP, dGTP, dCTP, dATP), 1 µL of both the forward and reverse primers, 1 µL of *Taq* polymerase and an additional 77µL of nuclease-free water.

Components used for PCR Reaction

DNA amplification was performed in PCR tubes comprising of the reaction mixture. Reaction mixture composed of the listed reaction mixture components (Table 2).

Table 2:- Reagents with their concentration and quantity used for single PCR reaction for NB1 and NB3 Sample

S.No.	Reagents	Final / working Concentration	Quantity
1.	Genomic DNA	50ng	2µl
2.	*Taq* Polymerase buffer (5X) + $MgCl_2$	1X	10µl
3.	dNTP's (10mM)	200 µM	8µl
4.	Forward Primer (10 µM)	0.5µM	1µl
5.	Reverse Primer (10 µM)	0.5µM	1µL
6.	Nuclease free water		77µL
7.	*Taq* polymerase (5U/µl)	1.25 µM	1µL
	Total		100µL

PCR amplification programme

PCR tubes, containing the master mix and DNA template, were thoroughly mixed and subjected to the PCR thermal profile outlined. The amplification process was held in a 96-well universal Gradient Thermal Cycler. The thermal cycling program includes an initial denaturation step of 95°C for 3 minutes. This was followed by a specific number of cycles, each consisting of denaturation at 95°C for 1 minutes , annealing at 52°C (based on the marker used) for 45 second, and extension at 72°C for 1.5 minutes. A final extension step was performed at 72°C for 5 minutes. The amplified PCR products was stored at -20°C (Table 3).

Table 3:-Thermal profile used for DNA amplification

Steps	Cycles	Temperature	Duration
Initial denaturation	1	95°C	3:0 min
Denaturation	35	95°C	1 min
Annealing	35	52°C	45 sec
Extension	35	72°C	1.5min

Final Extension	1	72°C	5:0 min
Hold		4°C	00

Gel Electrophoresis The assessment of PCR products was performed on a 3 percent agarose gel. To prepare the gel, 3 grams of agarose was mixed in 100 ml of 1x TE buffer and heated for 2-3 minutes until the agarose dissolved. After cooling, within this mixture 5 µL of ethidium bromide (EtBr) was added for visualization, and then this solution was poured into a gel casting tray with combs. The mixture was left to solidify for 20-25 minutes. After solidification of the gel, the samples were loaded into the wells. Then this whole electrophoretic apparatus with the samples is allowed to run under the application of 120 V power supply for almost 3 hours. After that the gel is removed from the apparatus and visualize under the U.V transilluminator for visible isolated DNA sample bands .

PCR products was checked along with a 1Kb DNA ladder was which is loaded to serve as a biomarker for determining the product size of ISSR primers amplified PCR products.

Note:- To check whether the PCR generated the desired size of interest of DNA fragment , agarose gel electrophoresis is employed for size separation of the PCR products. The size of PCR products is determined by comparison with A DNA ladder, which contains DNA fragments of known size, run on the gel alongside the PCR product.

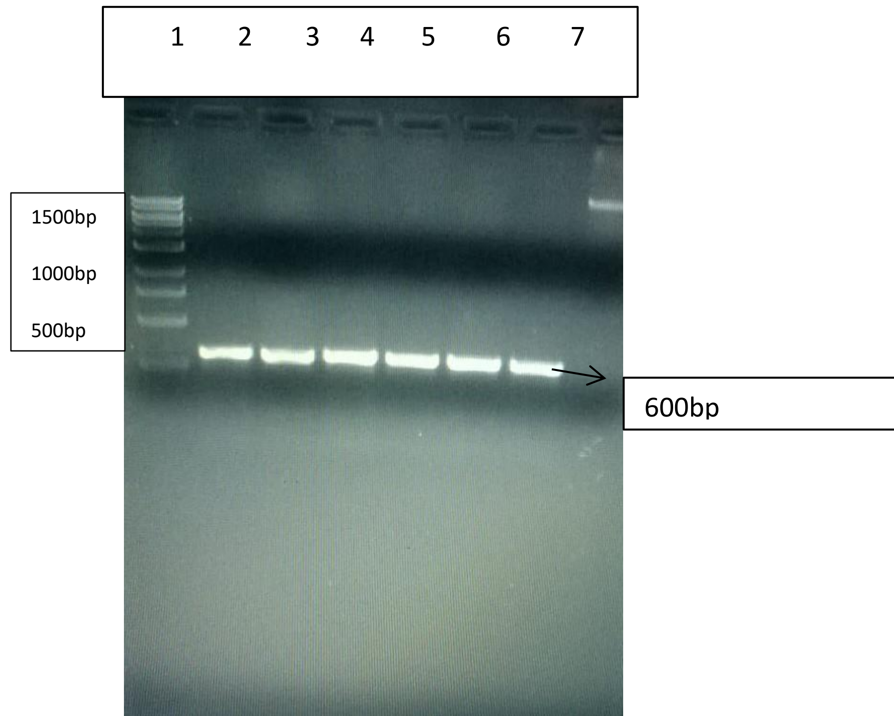


Fig 4:- 1lane -1 kb ladder, lane -2,3, 4,5,6,7 – NB1$_1$, NB1$_2$,NB1$_3$,NB3$_1$, NB3$_2$ and NB3$_3$ PCR product of isolated yeast of 600bp amplified by using ITS primer

Identification of yeast isolates

Identification of yeast upto species level was carried on the basis of standard cultured , morphological and physiochemical / biochemical tests , (Harrigan, W.F. and McCance, M.E.1982; Kurtzman CP, Fell JW,

Boekhout Tand Robert V ,2011b).

Culture characterization

Culture characterization of yeast isolates were done by streak culturing of isolates on YPD plates and incubated at 30°C or 48 hours and then the colonies were observed for following characterstics:
Shape : Shapes of the isolates colonies were rod, tubular or irregular.
Colour: colour was recorded as creamy , and slimy.
Surface : surface was recorded as smooth and slimy.

Morphological characterization

Morphological characterstics were determined by examination. To determine the morphology of yeast cells and reproductive type, the cultures were examined microscopically (Barnett et al,2000), vegetative cells were observed after 3 days of incubation at 30°C on YPD medium.

Slide preparation

A small drop of sterile deionized water was placed upon the center of slide , with proper aseptic technique ; small drop of these cultures was used to smear yeast samples (NB1 and NB3) with the help of inoculating loop. Then the slide was allowed to air dry and coverslip was placed onto it, water is wipe by tissue paper . All the slides with live yeast were then observed under the microscope in 100 times magnification,

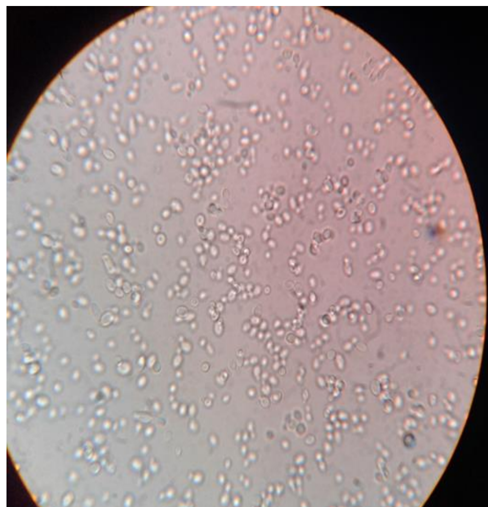


Fig 5:- cell morphology of isolated yeast strains (NB 1 and NB3) under microscope

Growth on solid medium

Morphology of cells of the selected isolates and their appearance on YPED agar media was examined. The medium was autoclaved at 121°C at 15psi and poured on Petridish and allowed to cool. After cooling the plates were inoculated with yeast strains (NB1 and NB3) separately and incubated at 30°C for 48 hrs. The following features of the appearance of cultures were recorded, texture , colour and surface of colonies.

Physicochemical characterization

Detection of H_2O_2 tolerance of selected isolated yeast by serial dilution of samples (10, $10,^{-1}$ 10^{-2},10^{-3},10^{-4}) then allowed to grow on YPD agar plates at specific concentration of hydrogen peroxide containing plates such as 0.8 mM was observed to ensure hydrogen peroxide sensitivity stress on strain. .

Detection of thermo tolerance

YPD plates were used for detecting thermo tolerance of selected isolated yeast. The medium was autoclaved at 121°C and 15psi and cooled, then allowed yeast strains(NB1 and NB3) growth on YPD culture plates at different temperature such as 30°C and 37°C which was observed for thermo tolerance of the isolated strains.

Glycerol test

YPD plates were used for detecting Glycerol tolerance of selected isolated yeast. The medium was autoclaved at 121°C , 15 psi and then cooled, after that 4ml of 4% glycerol is added and then serial dilution of samples (10, $10,^{-1}$ 10^{-2},10^{-3},10^{-4}) of isolated yeast was done then they are allowed to incubate at 30 °C for 48 hrs and then tolerance to glycerol concentration was observed.

Galactose test

YPD plates were used for detecting Galactose tolerance of selected isolated yeast. The medium was autoclaved at 121°C and 15 psi and cooled, then 200 µl of 20 % galactose is added and then serial dilution of samples (10, $10,^{-1}$ 10^{-2},10^{-3},10^{-4}) of isolated yeast strains NB1 and NB3 was conducted, then they are allowed to incubate at 30°C for 48 hrs and tolerance to galactose concentration was observed.

Growth at different pH in solid media

YPD agar containing solid medium was used for detecting the ability of the isolated yeast strains to grow at different pH. The medium was autoclaved at 21°C and 15 psi and cooled. After cooling the plates were inoculated by 48hrs oil yeast strain and incubated 30°C for 48 hours. The following features of the appearance of cultures were recorded, texture, colour and surface of colonies.

Fig 6:- Streak plate culture of NB3 and NB1 strains of isolated yeast at 30°C and 37 °C showing growth on Solid YPD agar plates after incubation for 48hrs ,

Physiochemical characterization

1. **Hydrogen Peroxide test**

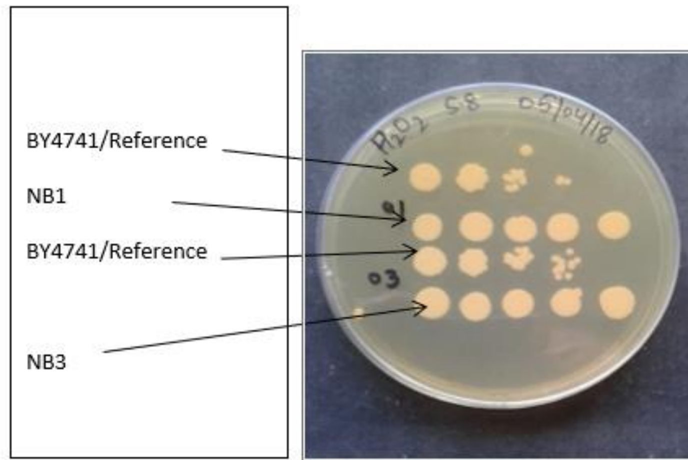


Fig:- 7 Effect of H_2O_2 Concentration on isolated yeast on YPD plate

2. **Thermotolerance test**

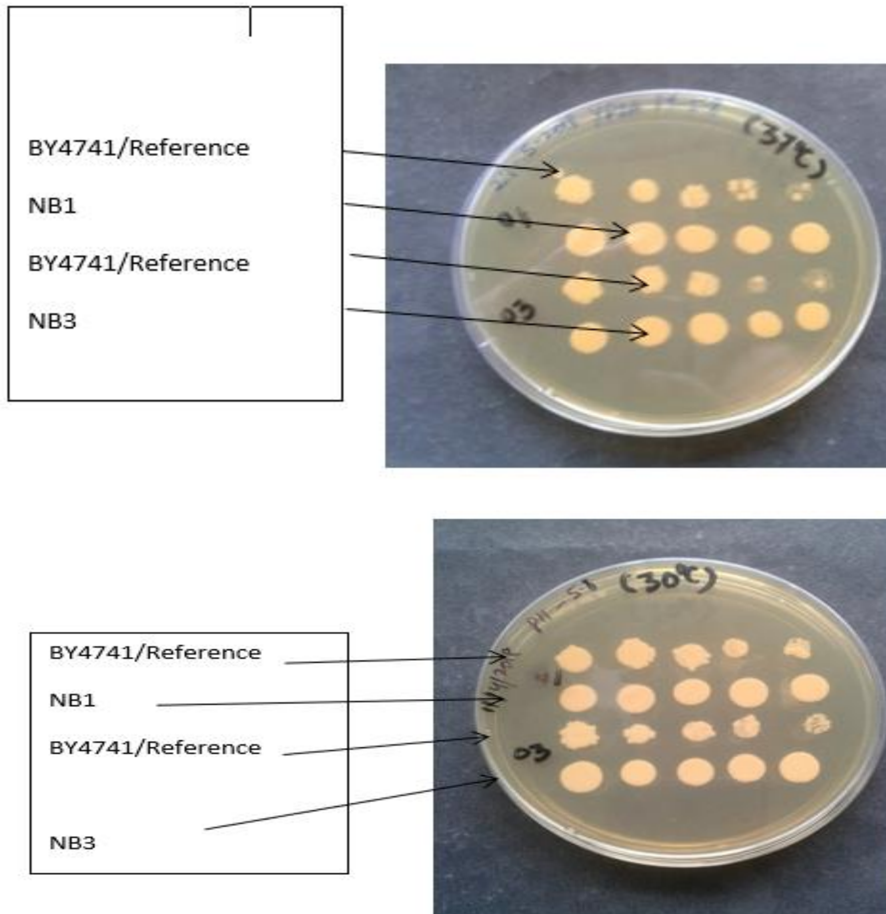


Fig:-8 Effect of different temperature (30°C and 37°C) on growth of yeast isolate on YPD

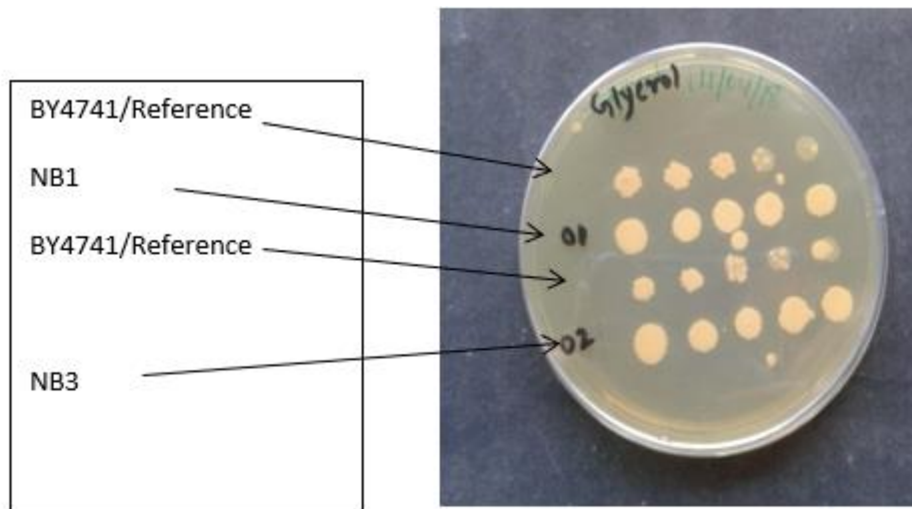


Fig:-9 Effect of glycerol concentration on isolated yeast in YPD plate Growth at different pH

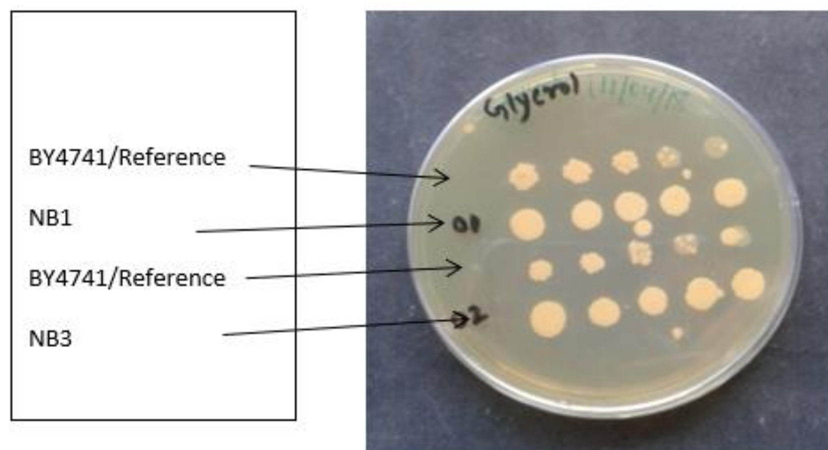
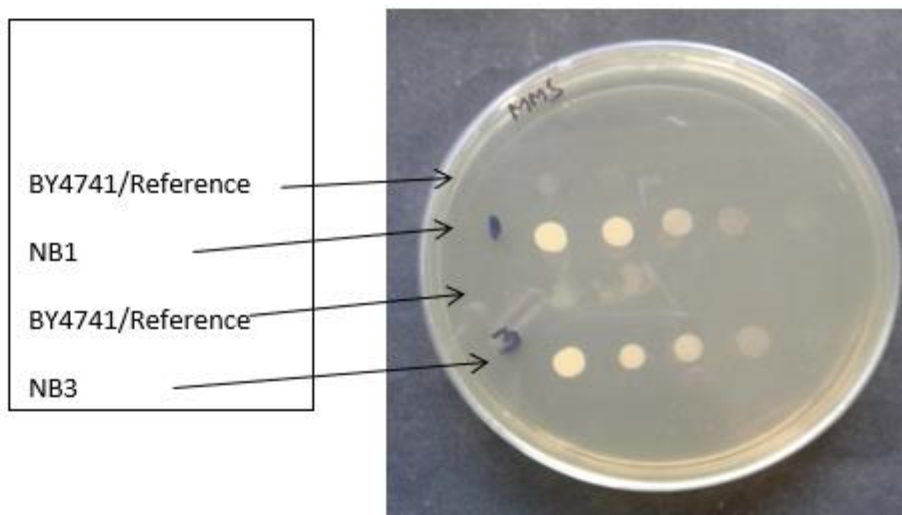


Fig 10: – Growth of isolated yeast at different Ph(PH5.8, 7,8) in YPD plates



Culture dilution method

1 ml of yeast culture was taken. Centrifugation was done at 3000rpm for 5 min at room temperature. Then Supernatant was discarded. After that, pellet was taken and washed with 1ml of autoclaved water. Again, centrifugation was done at 3000rpm for 5 min at room temperature. Once more, supernatant was discarded. Then Pellet was taken and dissolved in 500ul autoclaved water. Then dilutions were performed as per the dilution methods (serial dilution of samples (10, 10,-^{1} 10-^{2},10-^{3},10-^{4}) of isolated yeast strains NB1 and NB3 was conducted, then the culture plates are allowed to incubate at 30°C for 48 hrs.

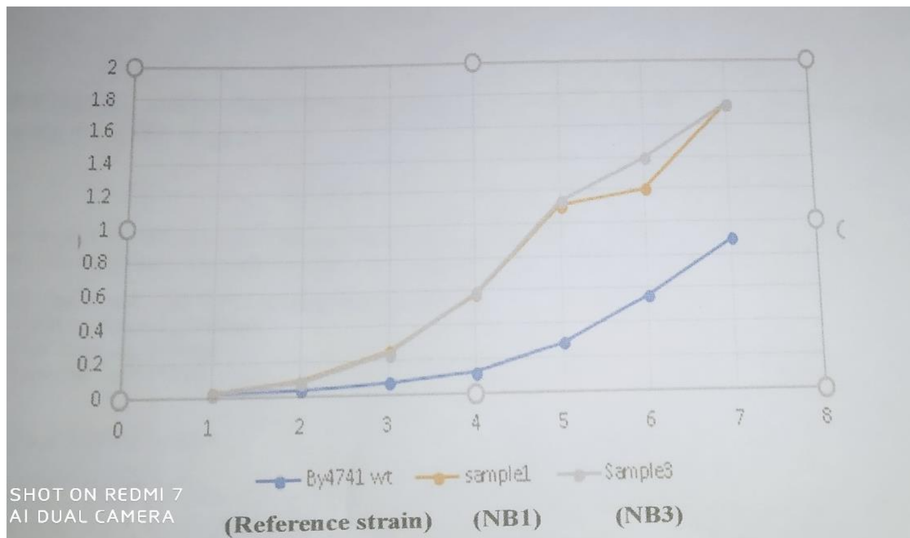
Growth Curve Analysis of Isolated (NB1 , NB3) yeast strains

Each of the two culture strains (NB1, NB3) with control (BY4741) was inoculated in 10ml of YPD broth. Then Incubation of 12 hrs was given in incubator shaker at 30°C.After 12 hrs each of 200 µl culture was added in a freshly prepared 50 ml of YPD broth for sub culturing .after that, the 2ml cultures were taken periodically after every 2 hrs of incubation for taking OD at 600nm (total duration of 12 hrs) in spectrophotometer.

Observation – Growth pattern of isolated yeast strains (NB1 and NB3) was observed and recorded.

Samples	0hrs	3hrs	4hrs	6hrs	8hrs	10hrs	12hrs
BY4741wt/ Reference strain (laboratory control sample)	0.02	0.04	0.07	0.123	0.287	0.557	0.894
Sample NB1	0.24	0.09	0.251	0.581	1.106	1.201	1.723
Sample NB3	0.2	0.082	0.237	0.593	1.132	1.391	1.726

Results: From this study it was concluded that both the isolated yeast strains sample 1(NB1strain) and sample 3(NB3 strain)displayed varying growth pattern , both of them are faster growing strains with a short life span.



Results:

Culture characterization of yeast isolates were conducted by streak culturing of isolates on YPD plates and incubated at 30°C for 48 hrs. Morphology of yeast cells was examined microscopically(Barnett et al'2000), vegetative cells were observed after 3 days of incubation of yeast cells culture at 30°C on YPD medium . Physiochemical characterization for detecting hydrogen peroxide tolerance of selected isolated yeast stains NB1 and NB3 by serial dilution of (10, 10,-^{1} 10-^{2},10-^{3},10-^{4})was Conducted, the culture plates are allowed to incubate at 30°C for 48 hrs. The samples were grown on YPD agar plates at specific concentration of hydrogen peroxide containing plates on 0.8Mm was observed to ensure hydrogen peroxide sensitivity stress on strain. Thermo tolerance noticed for tolerance of selected isolated yeast strains(NB1 and NB3) grown on YPD plates at different temperature and thermotolerance of the isolated strain was detected. For glycerol test , the glycerol tolerance of the isolated yeast strains (NB1 and NB3) was detected with respect to 4 ml of 4% glycerol concentration and then serial dilution of (10, 10,-^{1} 10-^{2},10-^{3},10-^{4})was Conducted, then the culture strains were incubated at 30°C for 48 hrs and tolerance to glycerol concentration was observed. Tolerance to galactose was detected for 200µl of 20 % galactose concentration with serial dilution of selected isolated yeast stains NB1 and NB3 by serial dilution of (10, 10,-^{1} 10-^{2},10-^{3},10-^{4})was done and culture plates were incubated at 30°C for 48 hrs , tolerance to galactose concentration was noticed. All these tests revealed that the isolated yeast strains were highly resistant at different pH conditions of YPD agar medium (pH= 5.8,7,8), higher concentration of H_2O_2, Glycerol, Galactose, methyl methane Sulphonate but has shown very little resistant in case of MMS drug concentration tolerance . When spotted with control BY474 1 Reference laboratory strain on YPD agar plates with incubation at 30°C for 48 hours. Growth curve analysis of isolated (NB1 and NB3) yeast strain shows that both the isolated yeast strains (NB1 and NB3) revealed varying growth pattern, both of them were faster growing strains with short life span.

Future Directions

Whole genome sequencing would be carried on isolates NB1 and NB3 and further they would be explored for their use in various industries.

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