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Harnessing Over Ripe Fruits For Isolation And Optimization Of Xylose Utilizing Yeasts And Analysis Of Its Fermentation Products

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Abstract
D-xylose nestled within plant hemicelluloses, is primary pentose sugar, comprising the lion's share of all carbohydrates derived from the hydrolysis of renewable plant biomass. Though glucose reigns in natural abundance, the fermentation of D-xylose offers a strategic avenue to bolster the yield of diverse fermented products. Despite their promise, hemicellulose sugars often remain underutilized, despite being more readily obtained from cellulose in higher yields and at a lower cost than glucose. Their conversion potential is vast, spanning the production of xylitol and other valuable chemicals. Among these, ethanol emerges as a frontrunner, holding substantial market potential. While considerable research has been devoted to isolating xylose-fermenting yeasts from various sources, this investigation focuses on isolation and characterization of yeast strains assimilating xylose from a spectrum of fruit samples. The isolates in our investigation were identified as Debaryomyces hansenii and Meyerozyma guilliermondii with 18S rRNA gene sequencing. Optimization investigations were performed to delineate the optimal conditions for yeast growth and fermentation, with pH 4.5, temperature 30°C and 5% substrate concentration yielding the substantial biomass and viable cell counts. Fermentation assays conducted utilizing xylose-enriched media resulted in substantial ethanol yields of 12.56% (S2) and 23.46% (S4), along with xylitol production of 50.85% and 51.05% respectively. HPLC analysis affirmed efficient utilization of more than 99% of the available xylose. Our fermentation studies promise a harvest of various fermented products, each holding potential that would have different applications in the production of bioethanol and xylitol, fostering in the innovation of sustainable bio refinery development.

Keywords: Biomass-to-energy conversion, *Debaryomyces hansenii*, *Meyerozyma guilliermondi*, Optimization

INTRODUCTION

Yeasts are the unicellular eukaryotic microbes which belong to the fungi kingdom. The most commonly known yeasts are *Saccharomyces cerevisiae* which is also known as Baker's yeast. At present, yeasts that have the capability to transform sugars derived from plant biomass into a diverse array of functional products are gaining much importance (Aristidou & Penttila, 2000). The most abundantly found sugar in plant biomass is glucose. Till now much of the work has been concentrated on the conversion of glucose. Apart from glucose, plant biomass comprises various other sugars including xylose, arabinose, mannose, galactose and rhamnose which are also present, with D-Xylose being one of the most prominent. D-Xylose, an aldopentose sugar derived from hemicellulose from plant biomass (Cadete *et al*., 2014). It is included in the dietary carbohydrates and also it is a part of sugar composition of cereals, pulses, fruits and vegetables like carrots, potato, peas, etc. Recent investigations have demonstrated that diverse microorganisms like *Escherichia coli* can efficiently transform the xylose into various value-added products includes xylitol and succinic acid, along with glucose, with better outcomes than those yielded from glucose alone. Xylose represents about 30-40% sugars recoverable from the plant biomass. Yeasts, which produce a multitude of fermented products from xylose, have been identified from a variety of sources, comprising decaying wood, wood-boring insects, rotten fruits, tree exudates and soil from fields of agriculture (Lorliam *et al*., 2013). Prominent xylose assimilating yeasts comprise strains of *Spathaspora passalidarum, Pichia stipitis, Candida shehatae, C. lignosa, C. tenuis, Pachysolen tannophilus, C. insectosa* and *S. arborariae* (Chandel *et al*., 2011). Among these, *S. passalidarum* and *Pichia stipitis* are recognized to be the most efficient ethanol producers (Agbogbo & Coward-Kelly, 2008; Hou, 2012). More yield and productivity can be seen when there is an effective conversion of both glucose and xylose. The aldopentose D-xylose accounts for approximately one-third of the total carbohydrate proportion

in lignocellulosic biomass. Xylose metabolism occurs via oxidoreductive or isomerase pathways and engineering microbes, with these routes essential for enhanced bioconversion and product yields. Favourable economics for large-scale processing of ethanol from lignocellulosic biomass needs optimized xylose utilization. Due to the growing demand for energy need, much of the interest has been in the production of renewable sources of energy. *Saccharomyces cerevisiae* is employed in the production of glucose from sugarcane juice and molasses to yield bioethanol, a renewable energy source. Sustainable ethanol production continues to remain a challenge since this technology is a consolidated industrial procedure. The pentose released by hydrolysis of hemicellulosic matter compensates around 30% of lignocellulosic feedstock. The pentose assimilating yeasts have potential for the production of high-value functional products. A multitude of investigations have recently concentrated intensively on *S. cerevisiae* genetic engineering and aimed to ferment both glucose and xylose for ethanol production (Jeffries, 2006). The yield achieved was maximum by xylose-fermenting species. Another investigation demonstrated that the pentose can be utilized to produce alternative compounds like lipids, iso-butanol, organic acids, hydrogen and biodiesel utilizing the yeasts (Carly & Fickers, 2018; Celinska & Grajek, 2009).

Apart from these compounds, the potential to harness these sugars in biorefinery systems has substantially enhanced due to the emergence of novel alternative products that offer significant overall value (Bansal & Mondal, 2000; Bolumar *et al.*, 2008; Yan *et al.*, 2021; Jeong *et al.*, 2023). The primary goal of our investigation is to isolate xylose fermenting yeasts that have potential industrial applications from the over-ripe fruits (Guava, Chikoo, Apple, Banana and Papaya) and characterized through morphological, biochemical and molecular studies. The optimization studies further analyze the optimum conditions for growth of these isolates in terms of pH, temperature, substrate concentration and incubation period for better yield and efficacy for the fermented products obtained in the form of ethanol and xylitol.

MATERIALS AND METHODS

Media and Chemicals

The constituents of media including Bengal agar, malt extract, yeast extract, Rose yeast nitrogen base, dextrose and peptone were procured from HiMedia Laboratories Pvt. Ltd, India. All other reagents were acquired from HiMedia Laboratories Pvt. Ltd, India, unless explicitly stated.

Isolation of Yeasts strains from over-ripe fruits

The overripened fruits were procured from the regional market in Davangere, Karnataka, India. The overripen fruits were chopped into small slices and 20g of each were resuspended individually in aseptic solutions of 2%, 5%, and 9% NaCl (sodium chloride). The culture flasks were maintained at ambient temperature (28°C) for 4-5 days. Followed by, the xylose fermenting yeasts were isolated from the fruit suspension using Peptone-Xylose media (Nweze *et al.*, 2021). The obtained isolates were saved for further characterization.

Isolation of pure culture

The test isolates were cultured in Rose Bengal agar medium supplemented with chloramphenicol to suppress bacterial propagation and incubated for 48 hours at ambient temperature (25°C). Following incubation, fungal colonies were examined on the plates, morphology features, for growth and pigmentation to characterize the isolates. The colonies were examined under the bright field microscope following lactophenol cotton blue staining to observe the test isolates' microscopic features.

BIOCHEMICAL TESTS FOR THE ISOLATES

Ascospore Formation

The test isolates were inoculated onto 5% malt extract agar (MEA) and incubated for 2–5 days at 25°C. Post-incubation, lactophenol cotton blue staining was applied to the yeast isolates and examined under a microscope for the occurrence of ascospores (Suh *et al.*, 2008).

Nitrogen assimilation test

The yeast carbon base medium formulated with incorporation of sodium nitrite, ammonium sulphate and potassium nitrate combined with 2% agar to facilitate the cultivation and metabolic investigations of the yeast isolates. The media was sterilized and dispensed into labelled petri plates. The freshly cultured test organisms were deprived of nutrients for 48 hours in saline (0.9%). Each culture was then inoculated onto the respective labelled plates. The plates were maintained at 25°C for 4-5 days and monitored for growth.

Sugar fermentation analysis

The fresh liquid cultures of test isolates were introduced into sterilized peptone water comprising sugar and Durham's tube. Each of the isolates was examined with lactose, dextrose, xylose, galactose and cellulose. The inoculated cultures were maintained for 5–7 days at 25°C and gas production was monitored.

Urease test

A small inoculum of freshly cultured test isolate was introduced into urease agar, which had been solidified in a slanted position. The inoculates were maintained at 37°C for 24 hours (Srividya *et al.*, 2023). The seeded cultures were monitored for colour change.

Germ Tube Test

For each test isolates, the germ tube test was conducted following the fungal identification guidelines provided by the Indian Council for Medical Research utilizing fetal bovine serum as recommended by the Indian Council of Medical Research (2019). A sterile loop comprising of 2-3 colonies were gently emulsified in the serum and kept at 37°C for 4-6 hours. The serum suspension was subsequently analysed under a microscope.

Molecular profile of Isolates

The yeast isolates were employed for genomic DNA extraction. DNA purified from every test isolate was amplified by employing 18s forward (5'-TCCTGAGGGAAACTTCG-3') and 18s reverse (5'-ACCCGCTGAACTTAAGC -3') primers that are specific to nuclear ribosomal small subunit (SSU) 18s rDNA. The amplified products were examined under ultraviolet transilluminator after being run on 1% agarose gel. Furthermore, PCR (polymerase chain reaction) products were gel-extracted and purified. The PCR product was sequenced uni-directionally using ABI 3130xl platform. The BLAST (basic local alignment) search from NCBI (National Centre for Biotechnology Information) was employed with the default values to identify the aligned sequences of each isolate by comparing them to nucleotide databases (Wang *et al.*, 2008; Camargo *et al.*, 2018).

OPTIMIZATION OF CONDITIONS FOR OBTAINED YEASTS

pH:

Yeast isolates were cultivated in liquid broth of pH 3, 3.5, 4, 4.5, 5 and 5.5 supplemented with 1% peptone, 0.5% yeast extract, 4% (w/v) molasses and 0.5% sodium chloride to assess the optimal pH. The same proportion of yeast cells that were actively growing were added to prepared media and incubated at 30°C for 4 days. The ideal pH for growth and maximum biomass production was found by employing a spectrophotometer for assessing optical density at 550 nm.

Temperature:

The yeast isolates were cultivated on a pH 4.5 liquid medium comprising 4% (w/v) molasses (previously identified as optimal), 0.5% yeast extract, 0.5% sodium chloride and 1% peptone in an effort to ascertain which temperature is optimal and which strains can thrive at 25°C, 30°C and 37°C. The aseptic media were introduced with the identical quantity of actively growing yeast cells and incubated for 4 days at 25°C, 30°C and 37°C. A spectrophotometer calibrated at 550 nm was employed to determine the optimal temperature for growth and maximal biomass production (Htet *et al.*, 2018).

Substrate concentration:

The yeast isolates were cultivated on liquid media with a pH of 4.5 (formerly identified as optimal) which included 2%, 4%, 8%, 12%, 16% and 20% (w/v) molasses, 0.5% yeast extract, 0.5% sodium chloride and 1% peptone to attain the optimal molasses concentration. These were seeded with the identical proportions of actively grown yeast cells and maintained at 30°C for 4 days. Optimum molasses concentration was determined utilizing a spectrophotometer by monitoring optical density at 550 nm (Modi *et al.*, 2018).

Incubation Period:

The strains of isolated yeast were cultivated in liquid media with a pH of 4.5 comprising 4% (w/v) molasses (earlier established as optimal), 0.5% yeast extract, 1% peptone and 0.5% sodium chloride to evaluate the duration of incubation sufficient for the yeast isolates produce the optimal biomass with the significant proportion of viable cells. The aseptic media were seeded with identical proportions of rapidly growing yeast cells and (formerly determined as optimal) for a period of 6 days at 30°C. Optical density was recorded at 550 nm. To quantify the number of viable cells (CFU mL^{-1}) on inoculation media, the broth was kept under agitation, periodically diluted to 10-6 times, and subsequently appropriate dilutions spread onto plates. Following centrifuging 5 mL of inoculation material for ten minutes at 5000 rpm, the supernatant was

removed. Subsequently, the pellet was dried in a heat drier at 105°C until the weight of it remained constant and then weighed to ascertain its dry cell mass. To construct growth curve of yeast isolates that relates cream cell mass mL^{-1}, OD to CFU mL^{-1}, dry cell mass mL^{-1} and duration of incubation, these data were used and subsequently graph was plotted. Utilizing this curve, the minimal incubation time required for optimal growth and maximal viable biomass production was computed.

Dry cell mass and CFU estimation:

To assess the dry cell mass and colony-forming units (CFU) after varying their incubation periods, inoculated molasses broth medium samples were collected in test tubes and meticulously combined utilizing a vortex. Subsequently, 5 mL of every culture broth was centrifuged at 4000 rpm for 10 minutes at 4°C, after which the supernatant was carefully removed. The resulting cell aggregates were then fully dried at 105°C until a constant weight was achieved, allowing for the determination of dry cell mass/ mL. For CFU/mL determination, cell suspensions obtained following various intervals of incubation, were uniformly combined via vertexing and serially diluted up to a dilution factor of 10^6. The density of cells was subsequently measured as CFU/mL utilizing the spread plate approach using YMA (Yeast Malt Agar) media. This involved evenly spreading the diluted cell suspensions onto the surface of YMA plates, followed by incubation under suitable conditions to allow for colony formation. The resulting colonies were counted and CFU/mL values were calculated based on the dilution factors and the number of colonies observed on the plates (Mamun-Or-Rashid *et al.*, 2022).

Fermentation Process:

To assess the fermentation of D-xylose, cultures were grown in an YP medium adjusted to pH 5.5 with hydrochloric acid (HCl). The medium was supplemented with 6% D-xylose in a total volume of 50 mL YP medium contained within a 250 mL Erlenmeyer flask. Cultures were agitated on a rotary shaker at 6000 rpm at a temperature of 30°C for a period of up to 24 hours. Following incubation, the fermentation broth underwent centrifugation at 8000 rpm for 10 minutes (Jairam Choudhary *et al.*, 2016). This step facilitated the separation of cellular components from the supernatant. The supernatants obtained were then utilized to ascertain the concentration of xylitol, ethanol produced and the quantity of xylose consumed.

High-Performance Liquid Chromatography (HPLC) analysis

High-Performance Liquid Chromatography (HPLC) was employed for the analysis, allowing for accurate quantification of ethanol concentration, xylitol concentration and xylose utilization. Ethanol and xylitol separation was conducted using an HPLC system consisting of a Waters UV/Vis HPLC system operating at 425 nm, equipped with a UV-VIS detector also calibrated at 425 nm and an injection valve featuring a 20 uL sample loop. 10 uL sample was chromatographically separated on a Chromolith NH2, column with dimensions of 10 cm x 4.6 mm I.D., 2 µm particles. The mobile phase, prepared by mixing water and acetonitrile in an 85:15 (v/v) ratio, was filtered through a 0.45 µm filter. The flow rate employed was 3 mL/min and the total runtime for the analysis was 15 minutes. Data integration was performed using Empower 3 software and the results were obtained by comparing the chromatographic profile with known standards (Lopez *et al.*, 2014).

RESULTS AND DISCUSSION

Yeast isolation from overripen fruits

The aseptic NaCl (saline) solutions at 2%, 5% and 9% were utilized to isolate xylose using yeasts from rotten fruits as shown in Figure 1. Out of the eight isolates, only four of them showed the growth of yeast colonies as revealed by their morphological features on the agar plates as illustrated in Figure 2. These four isolates were considered to possess yeast and were labelled as S1, S2, S3 and S4. The other four isolates failed to exhibit typical yeast colony features and were therefore omitted from further investigation. The capacity of yeasts to develop into aggregates is vital for their colonization, pathogenicity and sexual reproduction (Vallejo *et al.*, 2013).

Biochemical analysis

The conventional way of species authentication is by performing biochemical examinations. The sole carbon source consumption examination, metabolic product conversion and nitrogen assimilation test are some of the traditional tests to detect and categorize microorganisms (Pitt & Barer, 2012).

Ascospore Formation

In general, spores develop to endure harsh heat, malnutrition and other biological and physiological challenges. One characteristic that distinguishes the phylum Ascomycota is the production of ascospores. They are the meiotic cell division-produced in the gametic stage of the life cycle (Neiman, 2005). As illustrated in Figure 3, the S1, S2, S3 and S4 isolates demonstrated to have one to two evanescent ascospores upon microscopic examination of isolates, implying that these organisms are not representatives of the *Saccharomyces* or *Candida* genera (Lachance, 2011).

Nitrogen assimilation test

The isolates S1 and S4 were able to grow on potassium nitrate, but S2 and S3 saw poor growth on potassium nitrate as the sole nitrogen source. With ammonium sulfate, S1, S2 and S4 showed growth, but S3 showed poor growth proving to be better than nitrate and nitrite salts (Wickerham, 1946). With sodium nitrate, S2 and S3 showed considerable growth (Figure 4), but S1 and S4 showed poor growth (Table 1).

Sugar fermentation test

The capability of each isolate to ferment galactose, cellulose, glucose, lactose and xylose was examined. The test isolate S3 was able to ferment cellulose. S2 and S3 were able to ferment galactose. Each test isolate, with the exception of S2, had the potential to ferment glucose. The test isolates S3 and S4 were able to ferment lactose. All the isolates were able to assimilate and ferment xylose (Figure 5), thereby fulfilling the main objective of our study (Table 1).

Urease test

Human cells generate urea as a waste product, which by itself is relatively harmless. However, when certain bacteria produce urease, this enzyme breaks down urea into ammonia, which is toxic to human cells (Rutherford, 2014). For the detection of harmful or pathogenic yeast, it additionally serves as a presumptive test (McTaggart *et al.*, 2011; Marcos & Pincus, 2012). Each of the isolates appeared to be avirulent based on the outcomes of the biochemical profiling for the identification of the urease enzyme, which showed that none of the isolates contained urease, as affirmed by the unchanged color of the urea broth medium (Figure 6).

Germ tube test

The germ tube assay rapidly tests yeast isolates for their ability to produce germ tubes in serum, an important characteristic associated with filamentation, pathogenicity and distinguishing *Candida* species. Despite 4 hours of incubation at 37°C, neither of the four test isolates exhibited the occurrence of a germ tube (Figure 7). This demonstrated that each of the isolates was devoid of urease enzyme, and indicates the absence of pathogenic strains such as *Candida albicans* in the test isolates (Hilmioglu *et al.*, 2007; Silva *et al.*, 2011). Furthermore, the identification of these clinically non-pathogenic yeasts instils confidence in their safety profile for industrial applications, mitigating potential regulatory hurdles and safety concerns.

Molecular identification of xylose utilizing yeasts

Modern advancements in the development and improved accessibility of molecular techniques have revolutionized the rapid species-level identification of the microorganisms (Raja *et al.*, 2017; Ollinger *et al.*, 2020). Conserved 18s rRNA region in eukaryotes enables primer design for SSU-targeted molecular profiling of yeast isolates (Singh *et al.*, 2012). The agarose gel was employed for examining the outcomes of PCR utilizing the isolated genomic DNA. The NCBI's BLAST was used to analyse the acquired sequences. Tables 2 and 3 provide a report of the BLAST outcomes for the sequences from S2 and S4 (isolates that were selected for examination based on qualitative evaluations). The BLAST evaluation of the S2 isolate demonstrated more than 99% similarity with different sequences and the assessment of the phylogenetic tree highlighted higher degree of relatedness to the sequence of *Debaryomyces hansenii* with accession number NG_063361.1 (Table 2). The BLAST analysis of the S4 isolate showed more than 98% similarity with different sequences and the phylogenetic tree assessment affirmed its closer evolutionary proximity to *Meyerozyma guilliermondii* with accession number KJ126853.2 (Table 3). The isolation and characterization efforts transcended mere taxonomic identifications, providing valuable insights into the metabolic potential and functional attributes of the isolated yeasts.

OPTIMIZATION OF CONDITIONS FOR OBTAINED YEASTS

Standardization of pH, temperature and substrate concentration:

With the foundation laid in isolation and characterization, our focus shifted towards optimizing fermentation conditions to harness the full fermentative potential of the isolated yeasts. To determine the optimum

conditions for the growth of the yeast isolates S2 and S4, the isolates were cultured in liquid media with other requirements and appropriate incubation as mentioned in materials and methods. Using a spectrophotometer, optical density at 550 nm was monitored. Conditions such as optimum pH, substrate concentration and temperature for optimal yield were obtained (Rodrussamee *et al.*, 2011; Okamoto *et al.*, 2014; Nwuche *et al.*, 2018). From our study, maximum OD was recorded at 4.5 pH for both isolates S2 and S4 on 2nd, 4th and 6th day of incubation indicating 4.5 pH is optimal for these xylose-fermenting yeasts (Figure 8). Similarly, with respect to temperature optimization, maximum OD was recorded at 30°C for both isolates S2 and S4 on 2nd, 4th and 6th day of incubation (Figure 9). During substrate concentration optimization studies, no turbidity was observed at 10% substrate concentration for S2 isolate and 1%, 10% substrate concentration for S4 isolate. Maximum OD was recorded at 5% substrate concentration maintained at 30°C for both isolates S2 and S4 respectively (Figure 10). The comprehensive suite of tests not only validated their xylose-utilizing capabilities but also shed light on their metabolic versatility and adaptive mechanisms.

Dry Cell Weight:

The optimal incubation time necessary for maximal biomass production and higher proportion of viable cells by yeast isolates was assessed through various examinations. This facilitated the generation of yeast growth curves, illustrating the relationship between the duration of incubation and various correlates with critical parameters assessed in this investigation, including pH (3.0–5.5), temperature (20°C–40°C), and substrate concentration (1%–10%). From the studies, it was observed that CFU, wet cell weight/mL and dry cell weight/mL values increased to maximum till day 4 (considered to be optimum) and after which declined till day 6 for both isolates S2 and S4 as shown in Table 4. This is because the cells died rapidly as a result of waste product accumulation, high biomass accumulation and diminished nutrient levels. Both yeast isolates demonstrate a threshold cell population above which the death phase starts. Overall, it can be concluded that S4 isolate recorded better results qualitatively than S2 isolate with a maximum of 3 CFU/mL, 3.6 and 3 mg/mL wet cell and dry cell weight respectively observed on day 4 as shown in Table 5 and Figure 11. The optimization process unveiled pH 4.5, temperature of 30°C and substrate concentration of 5% as the optimal conditions, underscoring the importance of environmental factors in modulating yeast metabolism and fermentation kinetics. Through systematic experimentation, we delineated the optimal pH, temperature and substrate concentration conducive to maximal xylose fermentation efficiency.

FERMENTATION PROCESS

HPLC results:

In comparison with the standard xylose curve and maximum peaks observed for S2 and S4 isolates, approximately 99% of xylose was consumed as depicted in Table 6 (Figure 12). Similarly, in comparison with the standard ethanol curve and maximum peaks observed for both isolates, 12.56% and 23.46% of ethanol was produced, as depicted in Table 7 (Figure 13). Lastly, in comparison with the standard xylitol curve and maximum peaks observed for S2 and S4 isolates, 50.85% and 51.05% of xylitol was produced respectively as depicted in Table 8(Gong *et al.*, 1981; Lopez *et al.*, 2014). Outcomes of the investigation are tabulated in Table 8 (Figure 14). Our optimization studies (performed previously), not only maximized ethanol and xylitol yields, but also streamlined fermentation processes, enhancing efficiency and productivity.

CONCLUSION

In conclusion, our investigation depicts a holistic exploration of xylose-utilizing yeasts, spanning from isolation, characterization to molecular identification and optimization of fermentation conditions. The isolation of *Debaromyces hansenii* and *Meyerozyma guilliermondii,* coupled with their comprehensive characterization, optimization of fermentation parameters and integration of the isolated yeasts into industrial processes holds significant potential for bioproduction. Moreover, the non-pathogenic nature of the isolated yeasts alleviates safety concerns, facilitating seamless integration into industrial processes. The culmination of our endeavours holds varied implications for industrial biotechnology, offering a sustainable and efficient avenue for xylose utilization. Ethanol, a key biofuel and xylitol, a valuable platform chemical, emerge as prime targets for bioproduction, offering renewable alternatives to fossil- derived counterparts.

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List of Figures:

Figure 1: The fruits were suspended independently in sterilized solutions 2%, 5%, and 9% sodium chloride. (A) Apple, (B) Banana, (C) Chikkoo, (D) Guava and (E) Papaya

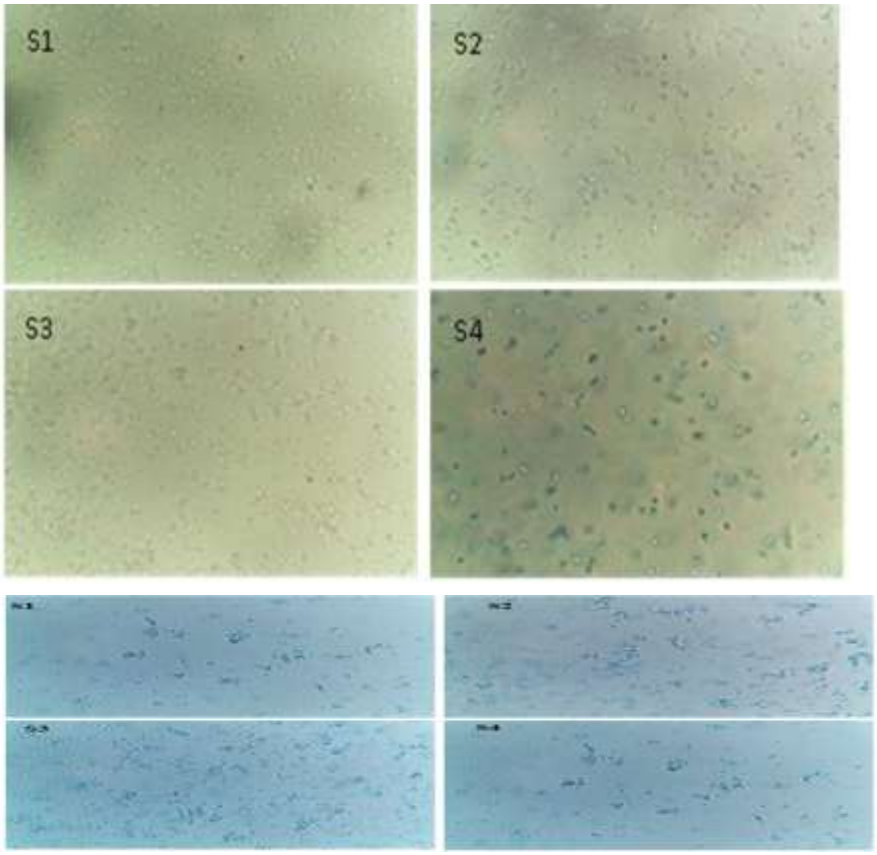

Figure 2: Microscopic observation of yeast cells grown on Rose Bengal agar media S1-S4 test isolates

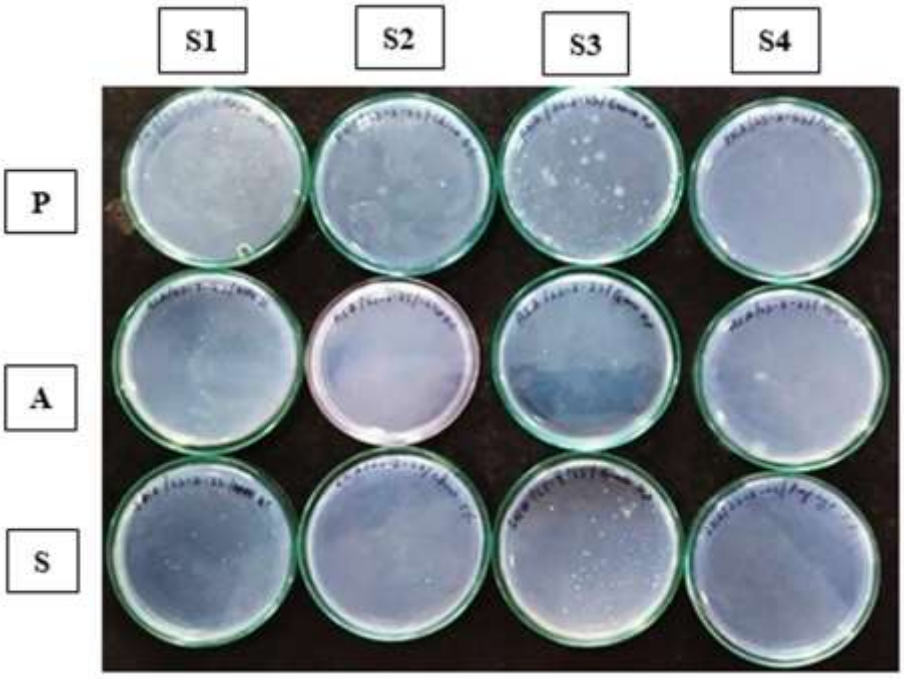

Figure 3: Ascospore formation- S1-S4- Lactophenol cotton blue stained cells

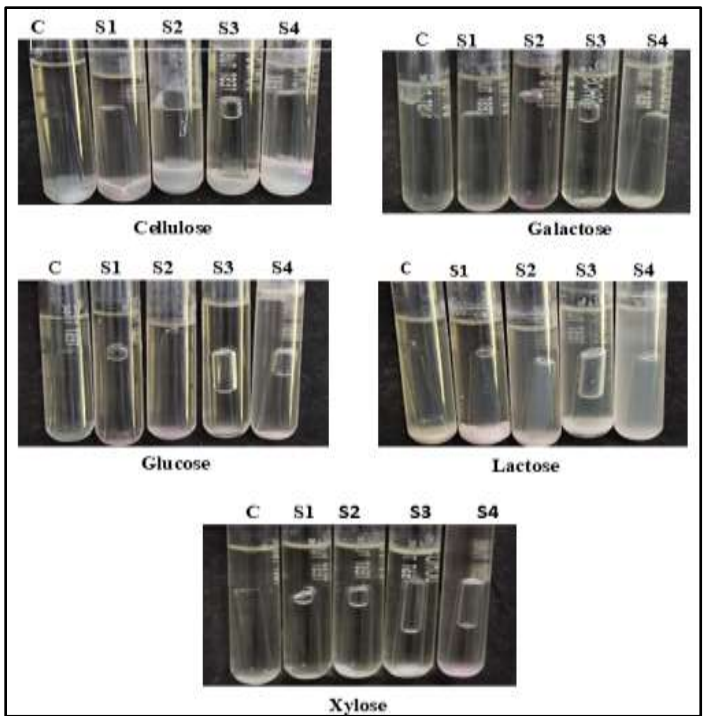

Figure 4: S1–S4 isolates- Growth on minimal media with their nitrogen resources. (P: Potassium nitrate, A: Ammonium sulfate, S: Sodium nitrite)

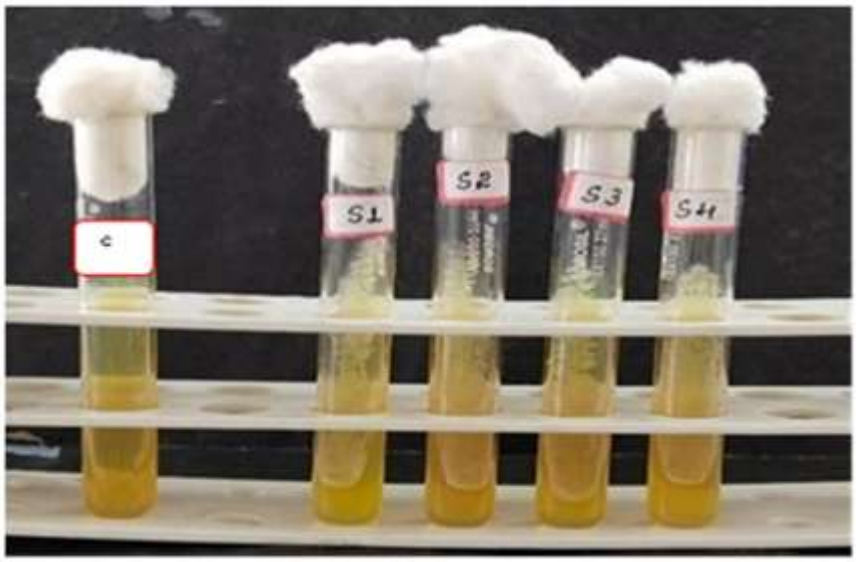

Figure 5: Outcomes of sugar utilization and fermentation analyzed in liquid nutrient media

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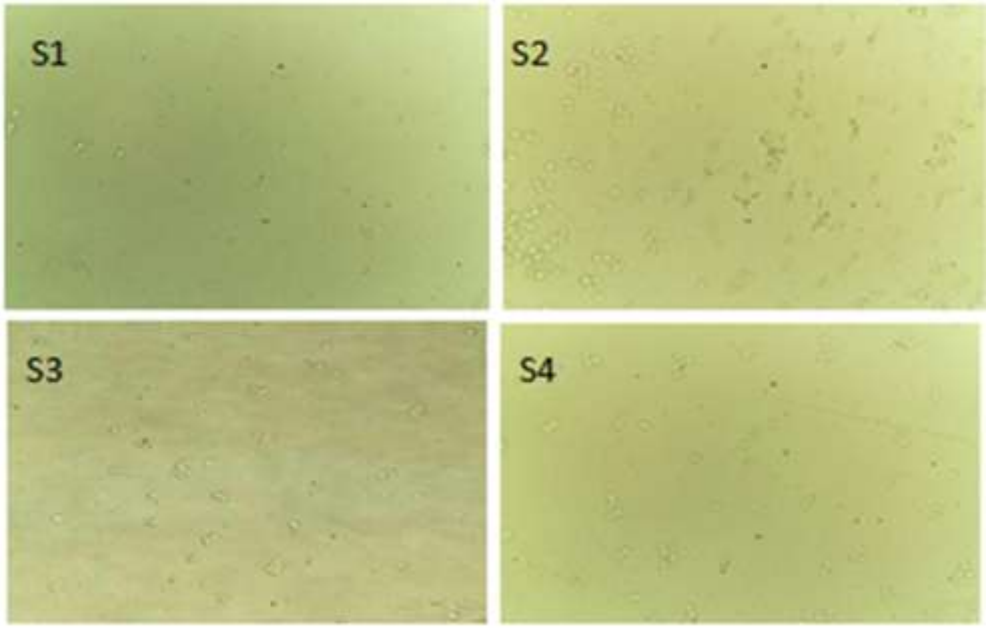

Figure 6: Results obtained from urease test (S1–S4 indicating the respective yeast isolates)

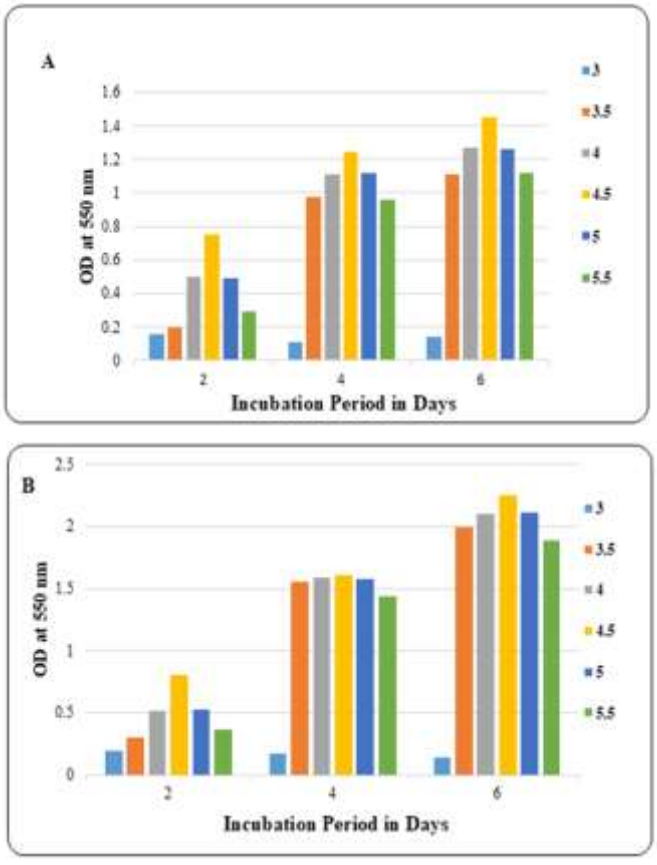

Figure 7: Results obtained from germ tube test (S1–S4 indicating the respective yeast isolates)

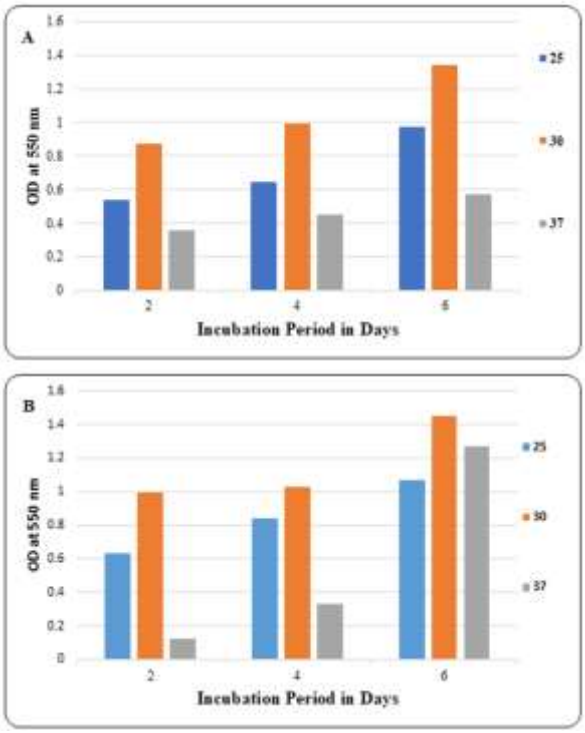

Figure 8: Optimization of pH of A) S2 isolate and B) S4 isolate

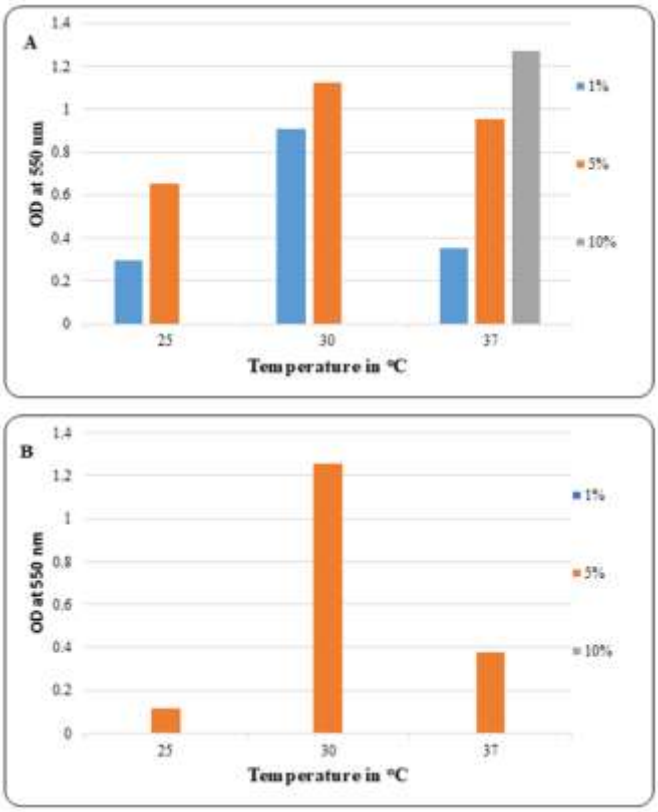

Figure 9: Optimization of temperature of A) S2 isolate and B) S4 isolate

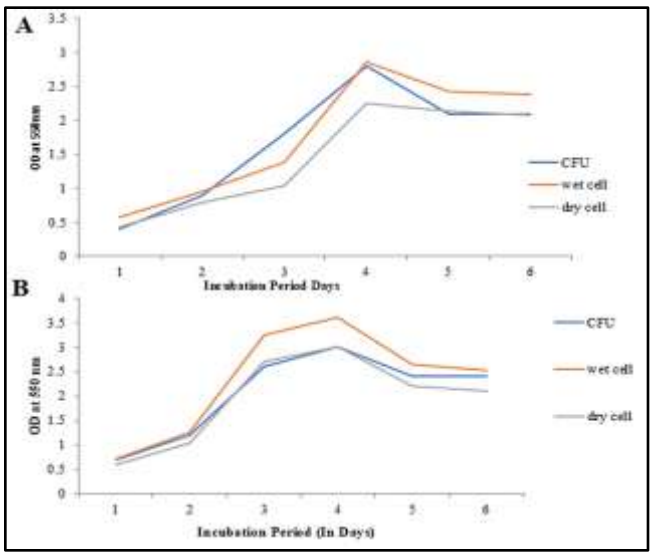

Figure 10: Optimization of substrate of A) S2 isolate and B) S4 isolate

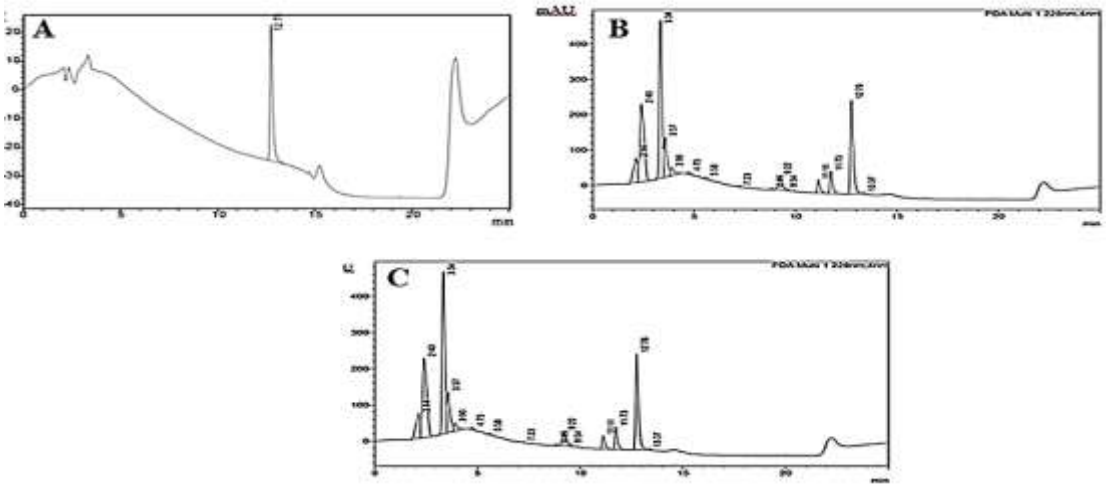

Figure 11: Relationship between incubation period, CFU, wet cell weight & dry cell weight for A) S2 isolate B) S4 isolate

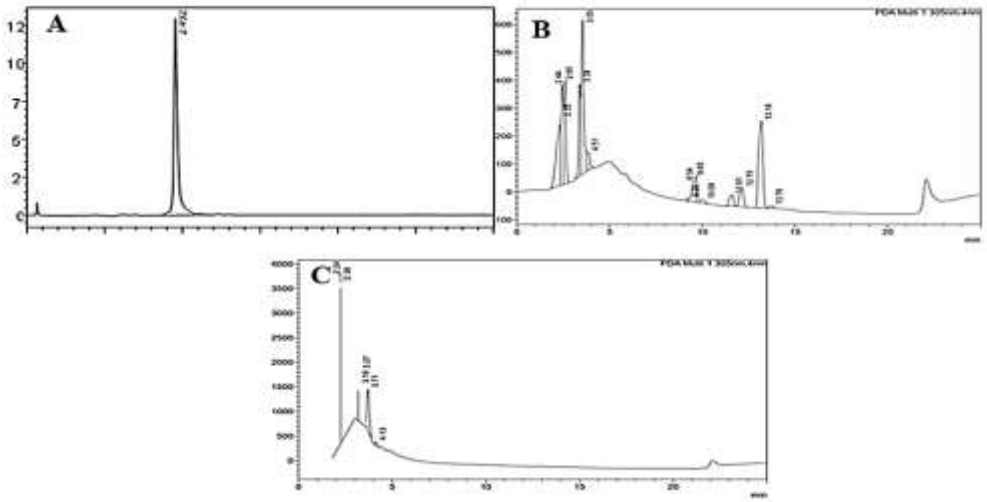

Figure 12: HPLC chromatogram of A) Standard Xylose B) Xylose curve for S2 isolate C) Xylose curve for S4 isolate

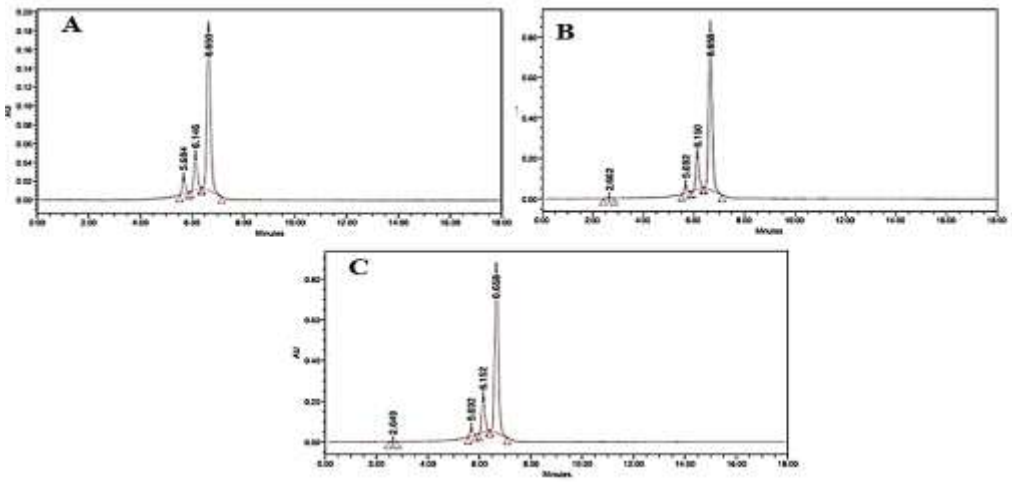

Figure 13: HPLC chromatogram of A) Standard Ethanol B) Ethanol curve for S2 isolate C) Ethanol curve for S4 isolate.

List of Tables:

Table 1. Results of nitrogen assimilation and sugar fermentation tests for the isolated yeasts S1–S4.

	Potassium nitrate	Ammonium sulphate	Sodium nitrite	Cellulose	Galactose	Glucose	Lactose	Xylose
C	-	-	-	-	-	-	-	-
S1	+	+	w	-	-	+	-	+
S2	W	+	+	-	+	-	-	+
S3	W	w	+	+	+	+	+	+
S4	+	+	w	-	-	+	+	+
+ : positive, – : negative, w : weakly positive								

Table 2: Summary of BLAST results of S2 sample

Sl. No.	Organism Name	Accession No.	% Match
1	*Debaryomyces hansenii* NRRLY-7426 18S rRNA gene	NG_063361.1	99.82%
2	*Debaryomyces hansenii var. hansenii* JCM 1990 18S rRNA gene	NG_063420.1	99.82%
3	*Debaryomyces psychrosporus* NCAIMY.01972 18S rRNA gene	NG_064957.1	99.82%
4	*Candida psychrophila* JCM 2388 18S rRNA gene	NG_063399.1	99.71%
5	*Debaryomyces prosopidis* JCM 9913 18S rRNA gene	NG_063491.1	99.53%
6	*Debaryomyces coudertii* JCM 2387 18S rRNA gene	NG_063486.1	99.53%
7	*Priceomyces fermenticarens* JCM 9589 18S rRNA gene	NG_063398.1	99.53%
8	*Meyerozyma guilliermondii* NRRL Y-2075 18S rRNA gene	NG_063363.1	99.47%
9	*Meyerozyma athensensis* CBS 9840 18S rRNA gene	NG_064893.1	99.47%
10	*Meyerozyma smithsonii* CBS 9839 18S rRNA gene	NG_064892.1	99.47%

Table 3: Summary of BLAST results of S4 sample

Sl. No.	Organism Name	Accession No.	% Match
1	*Meyerozyma guilliermondii* strain MguEd003 18S	KJ126853.2	98.80%

	ribosomalRNA gene		
2	*Meyerozyma guilliermondii* NRRL Y-2075 18S rRNA gene	NG_063363.1	98.80%
3	*Meyerozyma guilliermondii* CBS 9839 18S rRNA gene	NG_064892.1	98.76%
4	*Bispora christiansenii* 18S rRNA gene, isolate IMI 227584	AM279680.1	98.72%
5	*Candida sp*. KP-2012 18S ribosomal RNA gene	HQ916867.1	98.63%
6	*Meyerozyma guilliermondii* strain SW236 18S ribosomal RNA gene	KC178873.1	98.71%
7	*Debaryomyces hansenii* strain JHSa 18S ribosomal RNA gene	DQ534404.1	98.71%
8	*Pichia guilliermondii* strain gao1zhong2 18S ribosomal RNA gene	EF532297.1	98.79%
9	*Candida xestobii* 18S rRNA gene, strain JCM9569	AB013517.1	98.55%
10	*Pichia guilliermondii* strain JHSd 18S ribosomal RNA gene	DQ534403.1	98.63%

Table 4: CFU, Cream Cell Weight and Dry cell weight values for S2 isolate

Incubation period (In Days)	CFU (mg)	Cream/ Wet Cell Weight	Dry Cell Weight
1	0.4	0.58	0.43
2	0.9	0.96	0.79
3	1.8	1.38	1.04
4	2.8	2.86	2.26
5	2.1	2.43	2.14
6	2.1	2.39	2.08

Table 5: CFU, Cream Cell Weight and Dry cell weight values for S4 isolate

Incubation period (In Days)	CFU (mg)	Cream/ Wet Cell Weight	Dry Cell Weight
1	0.7	0.72	0.6
2	1.2	1.26	1.05
3	2.6	3.24	2.7
4	3.0	3.6	3
5	2.4	2.64	2.2
6	2.4	2.52	2.1

Table 6: HPLC results showing concentration of xylose consumed by yeast isolates S2 and S4

Sample	Initial (mg/100 ml)	Final (mg/100 ml)	% Utilised
S2	100	2.993641	**99.97006**
S4	100	2.291378	**99.97709**

Sample	Area	% Produced
S2	30151619	12.56
S4	56318231	23.46

Table 7: HPLC results showing amount of ethanol produced by yeast isolates S2 and S4

Table 8: HPLC results showing amount of xylitol produced by yeast isolates S2 and S4

Sample	RT	Area	Concentration	% xylitol production
S2	6.658	7853130	508.5	50.85
S4	6.656	7878771	510.5	51.05