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Immobilised *Wickerhamomyces Pijperi* Strain Ncim 3363 Potentials on Extracellular Invertase Production

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ABSTRACT

Fructose syrup is a product of the breakdown of sucrose using the invertase enzyme. Yeast was isolated from cashew apple wastes using Basal media. Qualitative screening for extracellular invertase production was carried out by the TTC staining technique, and the best isolate was identified molecularly. A quantitative assay was performed using the basal enzyme activity method. Optimisation of immobilised bead diameter size and other environmental parameters was conducted. The results showed that five yeast strains (W1, W2, W3, W4, and W5) were isolated. They presented varying colony characteristics on Basa agar. Isolate W2 gave a peak halo zone(22 ± 2.0) on qualitative assay and was identified.Subsequently, isolate W2 was characterized molecularly as *Wickerhamomycespijperi* strain NCIM 3363. In all, cell immobilised conditions significantly ($p < 0.05$) produced higher extracellular invertase enzyme (303 ± U/mL) when compared with free cells (195 ± U/mL). Immobilised yeast cells hold great promise in the industrial production of invertase.

INTRODUCTION

Invertase is an enzyme used for the production of an industrially important sweetener called fructose syrup. Enzymes are specific and break down known substrate into simpler compounds (William *et al.,* 2023; Raoufi *et al.,* 2023).Hydrolysis of sucrose, including its attached glycosides, to produce fructose syrup is facilitated by invertase enzymes (Genç, 2025 ; Nejatinejad & Fooladi, 2025;Taşar & Taşar, 2022). The sweetening characteristics inherent in the produced fructose syrup are unique when compared to sucrose (Chauhan *et al.,* 2016; Osiebe *et al.,* 2023;Mezaal *et al.,* 2025). Therefore, fructose syrup possesses better economic value than sucrose. Invertase enzymes are also referred to as β – fructofuranosidase with enzyme commission number (EC) of .3.2.1.26 (Toledo *et al.,* 2019). β-fructofuranosidase belongs to the GH32 glycoside hydrolase that encompasses greater than 370 enzymes of microbial and plant sources (Alberto *et al.,* 2004). It can further be stated that β-fructosidase refers to invertase of yeast origin whereas α-glucosidase are invertases produced by fungi (Nejatinejad & Fooladi, 2025). Although both of them hydrolyse sucrose to produce fructose syrup, β-fructosidase breaks down sucrose starting from the fructose end, whereas α-glucosidase starts sucrose breakdown from the glucose end (Nejatinejad &Fooladi, 2025).

LITERATURE REVIEW

As a matter of fact, invertase enzymes are known to be in high demand in food industries, biosensor companies and pharmaceutical industries (Chauhan *et al.,* 2016; Nejatinejad & Fooladi, 2025; Vitolo, 2021). In addition, fructose also plays a positive role in the management of diabetic individuals due to its ability to facilitate the absorption of iron in children (Linde *et al.,* 2012). However, fructose syrups were first produced by sucrose hydrolysis using chemicals (acids) but were later discouraged due to challenges that include: generation of unwanted by-products and reduced production efficiency (Antošová & Polakovič, 2001).Stepping up production of invertase to meet industrial demands requires a microbial approach to maintain environmental friendliness and improve efficiency by removing energy wastage on the production of by-products. Sucrose-utilizing yeasts have been isolated from various sources. Some fruits usually contain substantial amount of sucrose and tend to act as suitable substrates for invertase- producing microbes. For instance, Adou *et al.,* (2012) reported presence of sucrose in cashew apple of cashew tree (*Anacardium occidentale*). Invertase production efficiency by microbes can be influenced by some environmental factors such as pH and temperature. During invertase production, pH is a crucial parameter and its changes are sensitive to enzyme production (Nejatinejad & Fooladi, 2025). On the other hand, invertase structure is known to be affected by change in temperature and they are described to be temperature-dependent (Manoochehria *et al.,* 2020). However, the production of extracellular biomolecules has shown significant efficiency in using immobilised organisms when compared to free cells (Oyeagu *et al.,* 2021; Oyeagu *et al.,* 2018). Perhaps, immobilised cells were reported to record an increased yield of extracellular products when compared to free cells. This research therefore focused on the optimisation of invertase production using immobilised yeast isolated from fruit (cashew apple) wastes.

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MATERIALS AND METHODS

Sample Collection And Preliminary Isolation Using Enrichment Medium

A 1 % cashew apple waste was inoculated in a basal broth media (2 g/L of sucrose, 0.5 g/L NH4SO4, 3 g/L KH2PO4, 1.5 g/L yeast extract and 0.02 g/L MgSO4.7H2O) after sterilization at 1210 °C for 15 min at 15 psi. They were incubated at 30 0C for 24 h.

Isolation of Sucrose-Utilising Yeast

The 24 hr enrichment basal broth mixed culture (1 mL) was obtained, serially diluted, and streaked onto freshly prepared basal medium agar. The Petri dishes were incubated for 2 days at 30 0C. Yeast isolates were further purified by subculturing on basal media agar.The colony morphologies of isolated yeast were preliminarily identified based on their colony characteristics.

Screening (Qualitative Assay) For Extracellular Invertase Production

A 5 mL quantity of each yeast isolate containing 1.0 x105 cells /mL was inoculated on100 mL (basal broth media) using a 250 mL conical flask. They were incubated for one day at 30 0C.Thereafter, 0.2 mL of crude enzymes produced from each isolate was inoculated in wells made aseptically using 6 mm cork borer on a prepared basal agar media.The Petri dishes were incubated overnight in the dark at 30 0C. Petri dishes were then stained using TTC reagent (0.1% Triphenyl tetrazolium chloride in 0.5 M NaOH) and allowed to stand in the dark for 20 min before washing using acetate buffer (0.1 M) at pH of 5 (Praveen-Reddy *et al.,* 2010). Red colouration around the wells where crude enzymes were introduced indicates the presence of extracellular invertase enzymes.

Quantitative Assay For Extracellular Invertase Activity (Free Yeast Cells)

Basal broth media were used to cultivate 1.0 x106 cells /mL of each yeast isolate for 24 h at 300C. Thereafter, the 24 h culture was centrifuged (9,000 rpm) at 4 0C for 20 min, and the crude enzyme was recovered as supernatants (Nejatinejad & Foolad, 2025). A 0.4 mL aliquot of the supernatant was mixed with 0.4 mL of 1 % sucrose and 0.1 M (1.2 mL) acetate buffer at pH 5. The set up was allowed 30 min of incubation at 300C before pipetting 0.25 mL of the incubated supernatant into 3, 5 – Dinitrosalicyclic acid (DNS) reagent (1 mL) in a test tube to end the reaction(Miller, 1959). The test tube was boiled in a water bath for 10 min, and the optical density was taken using a spectrophotometer at 540 nm. A unit of invertase activity was defined as the amount of enzyme required to release 1 µmol of glucose/min under assay conditions.

Quantitative Assay For Extracellular Invertase Activity (Immobilized Cells)

Calcium alginate was prepared according to Oyeagu *et al.,* (2021) using sterilised 2 % (w/v) sodium alginate before adding it to the suspension of yeast cells (1.0 10^6 cells / mL). They were mixed and the resulting calcium alginate beads were cultivated in basal broth media for 24 h at 30 0C. After the 24 h incubation, the culture was centrifuged (9,000 rpm) at 4ºC for 20 min, and the crude enzyme was recovered as supernatants. Invertase production was estimated according to the formula of Nejatinejad & Foolad (2025):

Invertase enzyme activity(U/mL) =

$$\frac{[Product\ concentration\ (\mu mol\ /\ ml)] \times Total\ reaction\ volume}{Reaction\ time\ (min) \times Enzyme\ volume\ (ml)}$$

Molecular Identification of Best Yeast Isolate

The DNA of isolate W2 was isolated following the instructions on the Eukaryote DNA miniprep kit (Zymo Research). Agarose gel powder (0.9 %) for Polymerase Chain Reaction (PCR) and DNA was prepared in addition to the application of Internal Transcribed Spacer (ITS) gene with target set of primers, ITS F: TCCTCCGCTTATTGATATGS, ITS R: GGAAGTAAAAGTCGTAACAAGG. Thereafter, the ITS gene of isolate W2 was sequenced, and the National Centre for Biotechnology Information (NCBI) database was utilized for identification using BLAST. Mega 12 software was used to construct the phylogenetic tree.

Optimisation of Invertase Enzyme Production Variation of Carbon Source

A 2 % w/v solution of different carbon sources (glucose, fructose, sucrose and xylose) was used to determine invertase enzyme activity using *Wickerhamomyces pijperi* strain NCIM 3363.Suspended/ free cells and immobilised cells (2 mm bead diameter size) were all determined at 30 0C, pH of 5 using 1.0 x106 cells /mL.

Variation of Immobilized Bead Diameter

Various immobilised bead diameters, such as 2 mm, 3 mm, 4 mm and 5 mm, were examined for quantitative invertase enzyme production using *Wickerhamomyces pijperi* strain NCIM 3363. Free yeast cells (1.0 x106 cells /mL) were used as control. Other conditions (30 0C and pH 5) were maintained.

Variation of pH Values

Different values of pH (4, 5, 6, 7 and 8) were investigated for extracellular invertase enzyme production as stated above using free and immobilized *Wickerhamomyces pijperi* strain NCIM 3363.pH values were adjusted using 1N HCL and 1N NaOH (Oyeagu *et al.,* 2023)

Variation of Nitrogen Sources

Different nitrogen sources (peptone, yeast extract, urea and diammonium phosphate (DAP)) were investigated for production of extracellular invertase using free and immobilized *Wickerhamomyces pijperi* strain NCIM 3363.

Variation of Temperature Regimes

Various values of temperature (25 0C, 30 0C, 35 0C, 40 0C and 45 0C) were investigated for the production

of extracellular invertase enzymes using free and immobilized *Wickerhamomyces pijperi* strain NCIM 3363.

Statistical Analysis

All the experiments were performed in triplicates. Statistical analysis of the data obtained was carried out with ANOVA, and the means were separated using Least Significant Difference (LSD)at $p \leq 0.05$.

RESULTS AND DISCUSSION

Isolation of Invertase-Producing Yeast

Five distinct colonies observed from yeast growth on basal media were isolated (W1, W2, W3, W4, and W5) for further testing. Considering the colony characteristics presented (Table 1), all isolates formed entire margins. Isolate W1 was distinguished by yellow colonies and was suspected to be Pichia sp. It showed a smooth surface and raised elevation. Isolate W4 and W5 showed white colonies but differed in colony elevation and surface formations. Yeast isolate W4 had pulvinate elevation with smooth surface, whereas W5 isolate was recorded to have a rough surface and a raised colony. The two isolates (W4 and W5) were subsequently suspected to be *Saccharomyces* sp and *Rhodotorula* sp, respectively. Perhaps, forty per cent of the isolated yeast (W2 and W3) were suspected to be *Wickerhamomyces* sp. Isolates W2 and W3 showed white colonies with a smooth surface and raised elevations.

Qualitative Screening

When the isolates were qualitatively examined for invertase production, all isolates produced halo zones when stained with TTC. This may be attributed to the

Table 1: Characteristics of colony morphology on basal and primary invertase screening (TTC reagent staining halo zone) result

Isolate	Color of colony	Surface	Elevation	Margin	Enzyme halo zone (mm)	Suspected Yeast
W1	Yellow	Smooth	Raised	Entire	10.5± 2.0	*Pichiasp*
W2	White	Smooth	Raised	Entire	22 ± 2.0	*Wickerhamomyces*sp
W3	White	Smooth	Raised	Entire	13.5± 2.5	*Wickerhamomyces*sp
W4	White	Smooth	Pulvinate	Entire	9.5± 1.5	*Saccharomyces*sp
W5	White	rough	Raised	Entire	12 ± 2.5	*Rhodotorula*sp

2 g/L sucrose contained in the basal medium used for the invertase screening assay. It was believed that sucrose selectively allowed only yeast that can efficiently utilize it as a carbon source to grow. Hence, the hydrolysates of all tested isolates were concluded to contain crude invertase enzymes owing to the halo zones observed. Aside from the inclusion of sucrose in the basal media, the cashew apple waste may also have contributed to the successful isolation of an invertase-producing yeast from all the cashew apple wastes collected. Evidence (Adou *et al.,*2012) showed a significant presence of sucrose (between 2.3 g/L and 5 g/L) in cashew apple obtained from the cashew tree (*Anacardium occidentale*). It was also suggested that the sample (cashew apple) remains a source of invertase-producing yeast. Specifically, isolates W2 and W3, suspected to be *Wickerhamomyces* sp, had peak (22 ± 2.0 mm) and second-best (15.5± 2.5 mm) halo zone diameters, respectively (Table 1 and Figure1). Isolate W5, suspected to be *Saccharomyces* sp, had the least halo zone diameter and was assumed to have produced the least invertase enzyme based on the results obtained. Isolate W2 was the best isolate in the qualitative invertase enzyme assay and was chosen for molecular identification.

Molecular Identification

DNA isolation and sequencing of its 18S rRNA gene supported our earlier assumption and identified W2 as *Wickerhamomyces pijperi* strain NCIM 3363, with 98.9% similarity. The level of relatedness as obtained from phylogenetic studies (Figure 2) showed two taxa

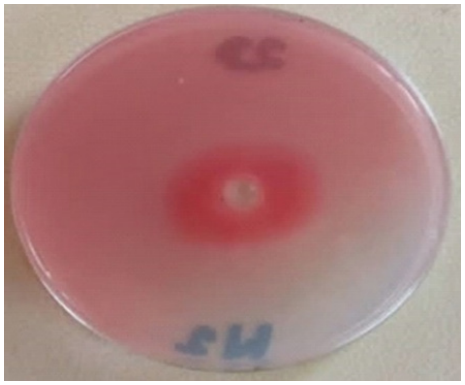

Figure 1: Primary invertase enzyme screening (TTC reagent staining halo zone) result of isolate W2

(*Wickerhamomyces pijperi* strain NCIM 3363 and Pichia pijperi strain CBS 2887) sharing the same ancestral node, indicating close relatedness. Whereas, other taxa (Candida solani strain CBS 1908, *Wickerhamomyces mucosus* culture CBS: 9334 and *Cyberlindnera japonica* CBS 7209) were not closely related to *Wickerhamomyces pijperi* strain NCIM 3363, as it did not share the same node with any other taxon except Pichia pijperi strain CBS 2887. *Wickerhamomyces pijperi* strain NCIM 3363 was obtained and optimised for invertase enzyme activity (quantitative analysis).

Quantitative Analysis

Secondary screening for invertase production was performed using 5 mL of 1.0 x105 cells /mL *Wickerhamomyces pijperi* strain NCIM 3363 in 100 mL basal media for both free cells and immobilised cells. The initial pH, temperature, carbon source and nitrogen source

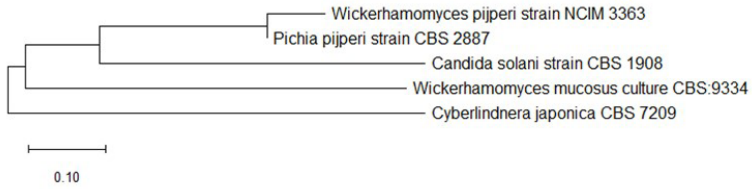

Figure 2: Phylogenetic tree of isolate W2 (*Wickerhamomycespijperi* strain NCIM 3363)

were maintained at 5 and 300C, sucrose and yeast extract respectively.

Variations on Carbon Sources

Four sugars (glucose, sucrose, xylose and fructose) were examined for invertase activity using free and immobilized *Wickerhamomyces pijperi* strain NCIM 3363 (Figure 3).Immobilised yeast cells had higher enzyme activities when compared to free cells. This may be attributed to high aggregation of cells in the immobilised cell condition. Oyeagu *et al.,* (2021) reported higher enzyme production potentials of fungi upon cell immobilisation. Sucrose and xylose sugars produced higher invertase activity than the hexose sugars.. A 2 g/L of sucrose as a carbon source revealed peak invertase production in both free cells (178 U/L) and immobilised cells (196 U/mL) at the pH of 5 and 30°C (Figure 3). Our findings on carbon source optimization substantiated the choice of sucrose sugar for invertase production by Nejatinejad and Foolad (2025).

Varying Immobilised Bead Diameter

Immobilised yeast bead diameters (2 mm, 3 mm, 4 mm and 5 mm) were investigated for enhanced invertase production efficiency using 2 g/mL sucrose, pH 5 and temperature of 30 °C. Results showed an increase

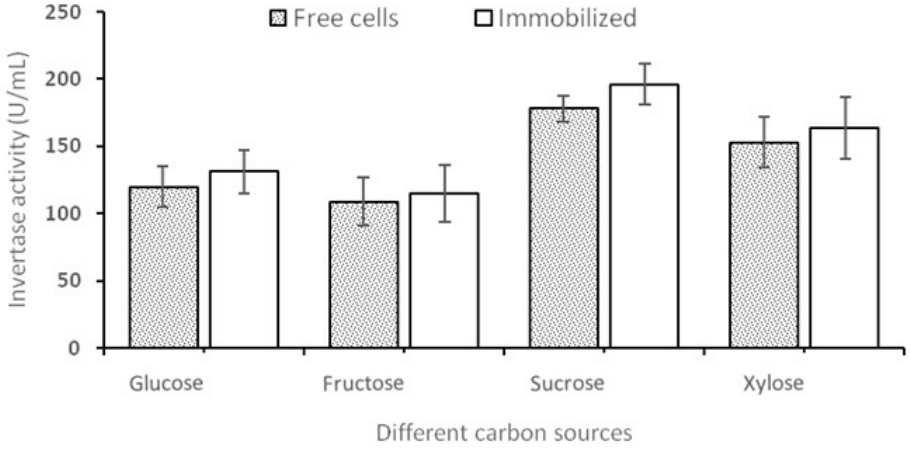

Figure 3: Production of invertase enzymes from different carbon sources using *Wickerhamomycespijperi* strain NCIM 3363

in invertase activity from 2 mm (186 U/mL) to 3 mm (202 U/mL), followed by declines to 151 U/mL at 4 mm and 114 U/mL at 5 mm (Figure 4). Smaller yeast immobilised bead sizes (2 mm) were believed to have experienced reduced mass transfer challenges owing to their small distances to diameters (Oyeagu *et al.,* 2018). Interestingly, it produced higher enzyme activity (186 U/mL) when compared to the free cells (165 U/L). A slight increase in yeast bead diameter (3 mm) resulted in peak invertase activity (202 U/mL). We suggested that this peak invertase activity by 3 mm yeast immobilised bead size may be attributed to higher cell aggregation in individual beads with improved nutrient and oxygen transfer across the gel bead matrix. Further increase in yeast bead diameter to 4 mm and 5 mm resulted in a decline in invertase activity. The low enzyme production

may be due to an increase in the calcium alginate beads matrix, which may have posed a serious challenge in the easy transport of nutrients and oxygen across the matrix. Oyeagu *et al.,* (2021) also reported a decrease in enzyme production when the bead diameter was increased to 4 mm and 5 mm, owing to reduced mass transfer to the fungi at the centre of the bead.

Effect of Varying pH Values

Invertase enzyme activity was investigated for immobilised and free cells using a temperature of 30 °C, 2 g/L sucrose, 3 mm yeast bead diameter, with varying values of pH (4,

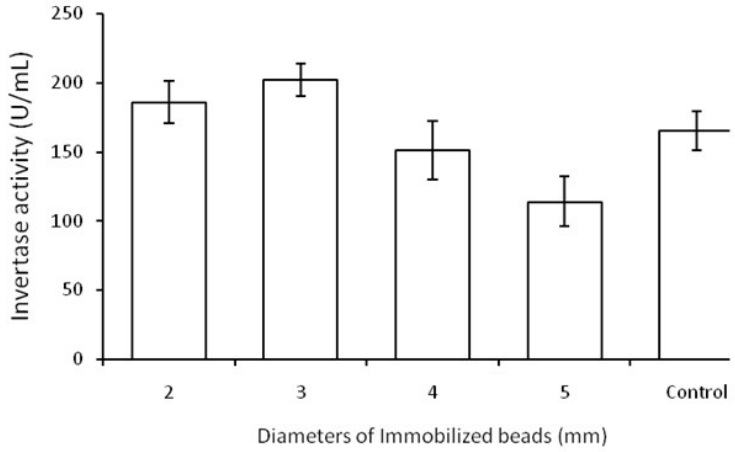

Figure 4: Invertase enzyme activity from varying bead diameters using immobilised*Wickerhamomycespijperi* strain NCIM 3363

5, 6, 7 and 8). Invertase enzyme activity increased in both free cells and immobilised cells as the pH increased from 4 (92 U/mL and 122 U/mL, respectively) to 6 (181 U/mL and 229 U/mL, respectively) before they declined (Figure 5). The least enzyme production was observed at a pH of 8 for both free cells and immobilised cells (75 U/mL and 91U/mL, respectively). On the whole, peak invertase production was recorded at pH 6, and immobilised yeast cells produced higher invertase when compared to the free cells. Nejatinejad & Fooladi (2025) recorded optimal invertase activity at a pH of 5 using free *Saccharomyces cerevisiae* PTCC 5209. In a similar work, the best invertase activity was recorded with Aspergillus niger at pH 5.5 ((Taşar and Taşar, 2022). Change in pH is known to be sensitive to microbial metabolite production, and immobilised cells tend to create resistance to toxic chemicals when compared to free cells (Elakkiya *et al.,* 2016).

Variations on Nitrogen Source

The initial pH for invertase activity across nitrogen sources was maintained at 6, while the temperature,

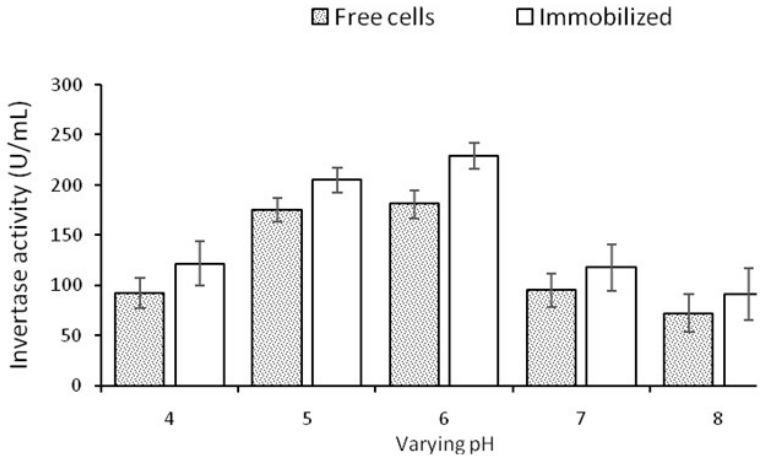

Figure 5: Production of invertase enzymes from varying pH values using *Wickerhamomycespijperi* strain NCIM 3363

carbon source, and yeast bead diameter were held constant at 300 °C, 2 g/L sucrose, and 3 mm, respectively. The best enzyme activity was recorded with peptone in both free and immobilised cells (198 U/mL and 229 U/mL, respectively) (Figure 6). Urea as a nitrogen source supported the least invertase activities for both free cells and immobilised cells (139 U/mL and 151U/mL, respectively).Similarly,Chauhan *et al.,* (2016) reported that peptone was the best nitrogen source for invertase activity in free cells. The results of Nejatinejad and Fooladi

(2025) differ, as urea was found to have supported the best invertase activity when studying the *Saccharomyces cerevisiae* PTCC 5209 efficiency. The discrepancy may be related to the differences in yeast samples and sources of isolation.

Temperature Optimisation

Immobilised yeast cells' invertase production increased as the temperature increased from 25 0C and showed a significant ($p \leq 0.05$) peak invertase activity at 35 0C (303 U/mL) before it declined (Figure 7). Free cells of *Wickerhamomyces pijperi* strain NCIM 3363 followed the same trend but had peak invertase production (195 U/mL) at a lower temperature (30 0C) when compared to immobilised cells. Very low invertase enzyme was produced at an increased temperature of 40 0C using free cells. However, immobilised cells surprisingly tolerated increased temperature (40 0C) by the production of invertase to higher levels of 231 U/mL (Figure 7). Manoochehria *et al.,* (2020) described the stability of invertase as temperature-dependent owing to its structure. This study provided evidence that the calcium alginate matrix conferred thermal resistance on invertase produced at 40 °C, compared with free cells. Free cells of *Wickerhamomyces pijperi* strain NCIM 3363 seem not to possess thermal resistance at 40 °C, hence had the best invertase at 30 °C. In another development, yeast invertase production mediated by silver nanoparticles exhibited peak invertase activity at a slightly lower temperature of 26.06 °C (Sowbarnika *et al.,* 2022).

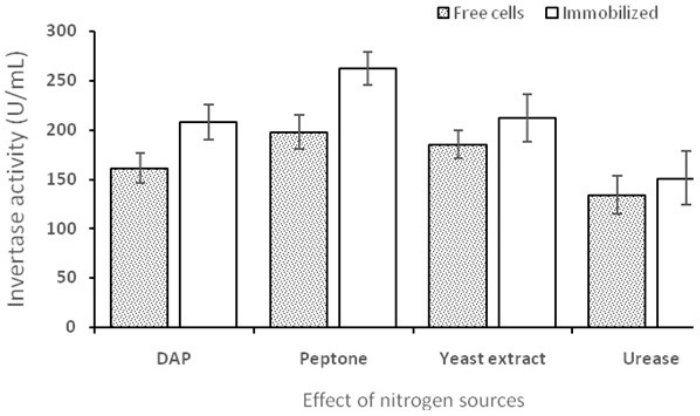

Figure 6: Invertase enzyme activity from different nitrogen sources using *Wickerhamomycespijperi* strain NCIM 3363

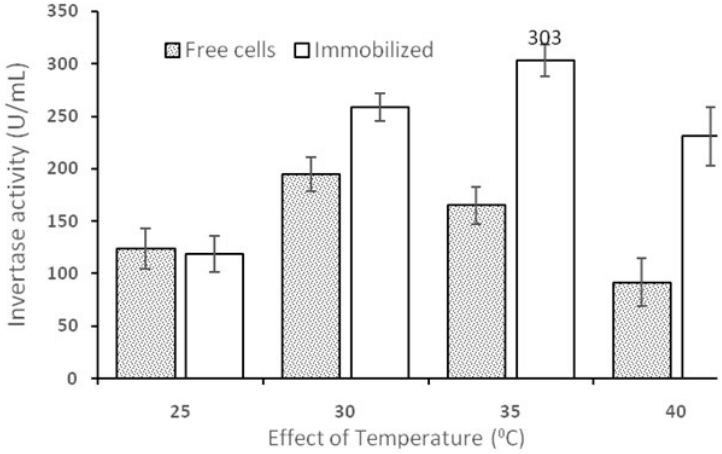

Figure 7: Production of invertase enzymes from varying temperature values using *Wickerhamomycespijperi* strain NCIM 3363

CONCLUSION

Cashew apple waste is noted as a reservoir for invertase-producing yeast. To the best of our knowledge, this was the first report of the involvement of *Wickerhamomyces pijperi* strain NCIM 3363 in invertase production. Sucrose, peptone and pH 6 were noted as the best carbon source, nitrogen source and pH for invertase production using free and immobilized *Wickerhamomyces pijperi* strain NCIM 3363. A 3 mm yeast bead diameter and 35 0C were most suitable for invertase activity using immobilized *Wickerhamomyces pijperi* strain NCIM 3363. On the other hand, the free *Wickerhamomyces pijperi* strain NCIM 3363 showed enhanced invertase production only at 30 0C. Overall, immobilised yeast cells were better than free cells in terms of invertase production in this study.

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