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PRODUCTION OF INVERTASE FROM *PENICILLIUM SPP.* UNDER SOLID STATE FERMENTATION AND ITS IMMOBILIZATION ON MAGNETIC NANOPARTICLES

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ABSTRACT

Invertase is an industrially significant enzyme that catalyses the hydrolysis of sucrose into glucose and fructose. The present work aimed to produce invertase from *Penicillium spp.* under solid-state fermentation, utilizing banana peel waste as the substrate, and to characterize and immobilize the enzyme using chitosan-coated magnetic nanoparticles. The partially purified invertase was obtained via ammonium sulfate precipitation. The stability of crude, partially purified, and immobilized invertase was assessed for various parameters such as pH, temperature, thermal stability, metal ions, and chemical inhibitors. Invert syrup was produced via continuous bioconversion. The optimal incubation period for invertase production was observed to be four days, resulting in a maximum invertase activity of 75 U/ml. The maximum activities of the crude and partially purified invertase were observed at pH 6, temperature 50°C, and metal ion KCl, while the chemical inhibitor EDTA enhanced the activity. Partial purification of the crude invertase with 70% ammonium sulfate yielded 49 U/ml, and immobilization on chitosan-coated magnetic nanoparticles resulted in a 59.01% immobilized yield. The maximum immobilized invertase activity was observed at 77°C. furthermore, thin-layer chromatography confirmed sucrose hydrolysis by *Penicillium spp*. derived invertase into glucose and fructose. Invert syrup production via continuous bioconversion was successfully achieved. The potential applications of invertase in the food industry, pharmaceuticals, confectionaries, and related fields.

Keywords: Invertase, Fruit waste, Solid state fermentation, *Penicillium spp.*, Magnetic nanoparticle, Immobilization

INTRODUCTION

Invertase is a glycosylated periplasmic protein and a cytosolic non-glycosylated protein **(Imaoka *et al*., 2002)**. Invertase also differs in molecular weight, e.g., intracellular invertase has a weight of 135000 Daltons, while extracellular invertase has a weight of 270000 Daltons **(Terauchi *et al*., 2000)**. A variety of microorganisms produce invertase using sucrose as the sole carbon source that can serve as an indicator **(Ikram-Ul-Haq and Ali, 2005)**. It has been reported that invertase is produced by both solid-state and submerged fermentation and that invertase is obtained by these fermentations.

SUCROSE + WATER ⟶ GLUCOSE + FRUCTOSE.

A wide range of microorganisms produce invertases. Commercially, invertase is biosynthesized by chiefly by yeast strains of *Saccharomyces cerevisiae* **(Mahendran *et al*., 2022)**. some of the fungi reported as invertase producers are the following: *Aspergillus niger* ATCC 20611, *A. niger* strain AN 166, *Aspergillus foetidus*, *Aspergillus oryzae* CFR 202, *Aspergillus awamori* GHRTS, and *Penicillium chrysogenum* **(Yun, 1996)**. some of these fungal strains have the capacity to produce two types of invertase, but the enzymatic activity is different because it depends on the carbon source and type of microorganisms **(Mahendran *et al*., 2022)**.

Invertases have gained widespread recognition as ubiquitous catalysts due to their environmentally friendly nature, cost-effectiveness, high specificity, and ability to operate under mild reaction conditions **(Hong *et al*., 2007)**. These enzymes hold significant importance as biocatalysts and find extensive utility across diverse fields, including biosensors, pharmaceuticals, chemicals, and food applications **(Bankar *et al*., 2009)**. Nonetheless, certain limitations persist, such as their relatively short lifespan and limited operational stability, issues that have been effectively addressed through the implementation of immobilization techniques. The process of enzyme immobilization enhances various crucial aspects, encompassing operational stability, catalytic activity, specificity, lifespan, productivity, structural integrity, and even reduction of inhibitory effects. This technique involves the attachment or integration of enzyme molecules onto or within substantial structures, accomplished through mechanisms like support binding, crosslinking, and encapsulation **(Ansari and Husain, 2012)**.

Among various nanostructures, magnetic nanoparticle (MNP) has a wide range of applications. They possess unique properties, namely superparamagnetic, low toxicity and biocompatibility **(Ashtari *et al*., 2012)**. These properties are used for a variety of potential applications such as immobilization of cells by enzymes, bioseparation systems, immunoassays, drug delivery and biosensors **(Botha & Black, 2000)**. MNP can serve as a highly useful catalyst support enabling the immobilization and magnetic recovery of the catalyst **(Baig and Varma, 2013)**. The MNP can easily be stabilized in a fluidized bed reactor for the continuous operation of enzyme **(Ashtari *et al*., 2012)**.

MATERIAL AND METHODS

Sample collection

The local fungal strains *Penicillium spp.* were isolated from the SRICEAS college campus and identified at the SRICEAS microbiology laboratory, Athwagate, Surat. All isolates were stored on potato dextrose agar medium slants at 4°C. The Isolation and purification of fungi were carried out using single spore and hyphal tip technique according to **E.A. & Atalla (2020).**

Culture media

The nutrient solution required for solid state fermentation was prepared by using yeast extract, 10g; $NaNO_3$, 10g; KH_2PO_4, 5g; $MgSO_4.7H_2O$, 1g; Distilled water, 1 litter; pH- 5 The potato dextrose agar consisted of potato infusion, 200g; dextrose, 30g and 500ml distilled water **(Ghosh *et al*., 2017)**.

Identification of fungal strains

Identification by observation of colonial growth as well as microscopic observation was performed using one drop of lactophenol cotton blue and loopful of fungal mycelia mixed with stains on grease free slide and this preparation was observed after placing coverslip on the slide and observe under 45X magnification.

Inoculum preparation

The spore suspension was used an inoculum in all studies by suspending spores of 72 h grown fungal culture into 10 ml of sterile distilled water containing 0.01% of tween 80. Spore count was performed using Neubauer's chamber.

Production of invertase under solid state fermentation using *penicillium spp.*

Solid state fermentation (SSF) was carried out in an Erlenmeyer flask of 250 ml, containing 20g of hot air oven dried solid substrates like banana peel powder,

which was supplemented with 10 ml nutrient solution (pH 5). Each flask was plugged with non-absorbent cotton and autoclaved at 121°C for 20 min. The flask was inoculated with 1 ml of fungal spore suspension and incubated for 96 h at 30°C under static conditions. Intermittent mixing maintaining sterile conditions was performed.

Extraction of crude enzyme

The crude enzyme was extracted after fermentation by adding 50 ml of sterile distilled water. The contents of the flasks were mixed thoroughly on a rotatory shaker (120 rpm) at 30˚C for 45 min. The content was then filtered with Whatman filter paper no.1. The filtrate was used as a crude enzyme solution and was analyzed for invertase activity and protein content **(Sangeetha *et al.*, 2004)**.

Enzyme assay

Invertase assay was performed as per the procedure, 2 ml of reaction mixture contained 0.6 ml of sodium acetate buffer, pH 5.0 with 1 ml of 5% sucrose solution was added. The tube was incubated at RT for 10 min and then 0.4 ml of crude enzyme was added to the mixture. The tubes were further incubated at 50°C for 30 min. The Reaction was terminated by keeping the tube in boiling water bath for 10 min and was analyzed for the presence of reducing sugar and protein content **(Sumner and Howell 1935)**.
Estimation of reducing sugar: After cooling equal the reaction mixture DNSA was used to determine reducing sugar. The color developed in each case was read at 540 nm using the UV-Vis spectrophotometer **(Sumner and Howell, 1935)**.
Estimation of protein concentration: protein concentration was determined by using the Folin-Lowry ciocalteau method. Bovine Serum albumin (Hi-media) was used as a standard & the colour developed was read at 660 nm **(Lowry *et al*., 1951)**.
One unit of sucrose hydrolytic activity was defined as the: amount of protein invertase required to liberate 1 µmol (0.18 mg equivalence) of reducing sugar (glucose and fructose) from sucrose/min. under the assay conditions. it can be calculated by the following equation:

Invertase activity (units/ml) = 0.1 mg/ml glucose concentration × dilution factor/ aliquot

Partial purification by ammonium sulfate precipitation

Partial purification of invertase produced under SSF conditions was performed by fractional precipitation of crude enzyme using ammonium sulfate at 50%, 60% and 70% saturation. Solid extra pure ammonium sulfate was added to the crude enzyme for 20 % saturation and incubated at 4 °C for 1 h. After centrifugation at 10,000 rpm for 20 min. at 4˚C, the precipitates formed were dissolved in a minimum volume of 0.1 M phosphate buffer (pH - 6.0). Further ammonium sulfate was added to the supernatant to bring it to 30% saturation. After incubation at 4 °C for 1 h. it was again centrifuged. Similarly, the process was repeated for 60% and 70 % saturation. The precipitates after each step were dissolved in a minimum volume of 0.1 M phosphate buffer (pH - 7.0). Enzyme activity and protein concentration were assayed in the supernatants and precipitates obtained after each step **(Dhananjay & Mulimani, 2008)**.

Qualitative analysis of glucose, fructose and sucrose

The Qualitative analysis of oligosaccharides by thin layer chromatography (TLC) using the solvent systems chloroform: methanol: water (30:20:4). The products were visualized by heating the plates after spraying 5% sulfuric acid in ethanol. 0.1M sucrose, 0.1M glucose, 0.1M fructose were used as control **(Shamly *et al*., 2014)**.

Preparation of magnetic nanoparticles

Magnetic Fe_3O_4 nanoparticles were prepared by co-precipitation of Fe^{2+} and Fe^{3+} ions, followed by a chemical co-precipitation method with some modifications **(Pan *et al*., 2009)**. While 50 ml of 0.3M ferric and ferrous (molar ratio 1:1) solution in deionized water was vigorously stirred, 10 mL of ammonium hydroxide solution (25%) was added then 10M NaOH solution was slowly added, during the processing, pH and temperature were maintained at about 10 and 70 °C, respectively. After 30 min, the suspension, which was black, was cooled to room temperature with mechanical stirring. Finally, the resulting magnetic Fe_3O_4 particles were collected magnetically by a permanent magnet and washed several times with deionized water to remove the excess base till the pH reached 7. The reaction was as shown in below equation **(Qin *et al*., 2015).**

$$Fe^{2+} + 2Fe^{3+} + 8OH^- \rightarrow Fe_3O_4 + 4H_2O$$

Preparation of chitosan magnetic nanoparticles

A Specified amount of chitosan powder (0.8 g for 2% chitosan beads or 1.6 g for 4% chitosan beads) and 5 g of dried magnetic powder were suspended in 40 ml 5% acetic acid solution. The mixture was stirred to completely dissolve the chitosan and distribute the magnetic powders uniformly in the solution. This solution (the aqueous phase) was then poured into 260 mL dispersion medium (the oil phase) composed of mineral oil, petroleum ether, and an emulsifier (span 80, 10 ml). The volume ratio between mineral oil and petroleum ether was chosen according to the content of chitosan at 230:30 and 245:15, for the 2% and 4% chitosan beads, respectively. Upon emulsification for 10 min, the crosslinking reagent (25% aqueous glutaraldehyde) was added in. The amount of crosslinking reagent was examined from 4 ml to 16 ml. After adding the first half of the crosslinker, the reaction was continued for 1 h. The second half was added in thereafter and the reaction was held on for 2 h. When the reaction was stopped, the beads were separated from the oil phase via centrifugation. The beads were then thoroughly washed successively with petroleum ether, ethanol, and distilled water. During the course of washing, the beads can be recovered using a centrifuge or a magnet. magnetic nanoparticles were easily separated by a permanent magnet **(Saravanakumar *et al*., 2014**).

Characterization of magnetic nanoparticles

Preliminary characterization

The morphology of magnetic nanoparticles and chitosan magnetic nanoparticles was characterization using a light microscope

Separation of magnetic nanoparticles

Magnetic separation can be simply achieved by placing a permanent magnet close to a suspension containing magnetic nanoparticles. Using horizontal low gradient magnetic field (HLGMF) systems, high magnetic forces, implying fast separation and low magnetic nanoparticle losses, can be achieved **(Moeser *et al*., 2004)**.

Particles size distribution of magnetic nanoparticles

Dynamic light scattering (Malvern, UK) was used to determine the average particle size of the synthesized magnetic nanoparticles. The particle size distribution and zeta potential of the prepared magnetic nanoparticle were determined by the DLS technique using Zetasizer Nano ZS 90 (Malvern Instruments Ltd., UK) within the range of 0.1–10,000 nm at a scattering angle of 90° and 25° at Sardar Vallabhbhai National Institute of Technology (SVNIT), Surat. For the hydrodynamic diameter measurement, 1 ml of the sample was transferred into a disposable plastic cuvette, and automatically equilibrated in the instrument for 2 min. The data were recorded in triplicate. For the zeta potential analysis, 1 ml of the sample was injected into the zeta cell, and the measurements were repeated three times after equilibration.

Enzyme immobilization

Since there are free aldehyde groups in the cross-linked magnetic chitosan beads, enzyme immobilization was carried out by, simply soaking the beads in an enzyme solution. Prior to soaking, the beads were rinsed using sodium phosphate buffer (pH=5) several times. The beads were then incubated in sodium phosphate buffer for an hour, followed by separating the buffer from the beads by magnet. A preset number of beads was weighed and incubated with invertase at 1 g beads per 10ml solution and incubated at 4 ˚C for more than 24 h on a tube rotator. The beads were then recovered using a magnet and washed with sodium phosphate buffer several times. The immobilization or co-immobilization efficiency was determined by measuring the remaining enzyme activity in liquid phase and the activity of beads after immobilization **(Pan *et al*., 2009)**.

Yield of enzyme immobilization

Technically, the yield of enzyme immobilization can be determined in terms of expressed activity or protein content. Here, the former was selected for the calculation of the recovery activity, which is important for the evaluation of immobilization performance. Immobilization yield was determined in terms of expressed activity (YI) according to below equation **(Silva *et al*., 2012)**.

$$\mathbf{YI = A_{ini} - A_{fin} / A_{ini} \times 100}$$

where A_{ini} and A_{fin} are enzyme activities (U/ml) in the supernatant (soluble enzyme) before and after immobilization, respectively; YI is yield of immobilization.

Characterization and stability of crude, partially purified and immobilized invertase

Effect of pH on invertase activity and stability
The optimum pH of the crude and partially purified invertase was determined by incubating invertase in 0.1M citrate buffer (pH=4), 0.1M sodium phosphate buffer (pH=5), 0.1M sodium acetate buffer(pH=6) at 50ºC the pH effects on invertase activity were assayed at pH values ranging from 4 to 6 for 30 min. To determine pH stability, the crude and partially purified invertase was preincubated at 50ºC for 24 h at pH 4 to 6 **(Martínez-Villaluenga *et al*., 2008)**.

Effect of temperature on invertase activity and stability

The optimum temperature of crude, partially purified and immobilized invertase was determined in the temperature range of 50-70 ºC in 0.1M sodium acetate buffer at pH 6 for 30 minutes. The invertase activity was determined under standard assay conditions. To determine temperature stability, the crude and partially purified invertase was pre-incubated at 50-70ºC for 24 h at pH 6 **(Martínez-Villaluenga *et al*., 2008)**.

Effect of different metal ions on invertase activity

The crude and partially purified invertase was mixed with 0.2M concentration of various salts such as $CaCl_2$, $ZnSo_4$, and KCl for 30 min. at 50 ºC at pH 6 before adding the substrate and subsequently invertase activity was determined. To determine metal ion stability, the crude and partially purified invertase was pre-incubated at 50ºC for 24 h at pH 6 **(Madhan *et al*., 2010)**.

Effect of different chemical inhibitors on invertase activity and stability

The crude and partially purified invertase was mixed with 0.2M concentrations of different chemical inhibitors such as EDTA, H_2SO_4, and $HgCl_2$ for 30 min. at 50ºC pH 6 before adding the substrate and subsequently invertase activity was determined. To determine chemical inhibitor stability, the crude and partially purified invertase was pre-incubated at 50ºC for 24 h at pH 6 **(Masse *et al*., 2008)**.

A continuous bioconversion reactor system was designed at the laboratory level for the continuous production of invert syrup using immobilized invertase.

The system included the main components of the column, substrate reservoir, and production collector. The columns were filled up with magnetic nanoparticles-chitosan complex immobilized enzyme and glass wool. The substrate reservoir was filled up with 0.1M phosphate buffer, 5 % sucrose solution, pH 6 and flow rate was 0.82 ml/h. and fit at the top of the column. The product collector was attached at the bottom of the column and collected the invert syrup product. The whole system was place in water bath at a constant temperature of 50°C. after setting up the system, the flow rate and yield of invertase syrup formation were investigated **(Koli and Gaikar, 2017)**.

RESULTS AND DISCUSSION

Identification of fungi

The microscopic analysis revealed key morphological features consistent with the *Penicillium genus*, notably the presence of conidiospores borne on conidiophores. These conidiophores exhibited branching with finger-like projections, which is a characteristic trait of *Penicillium spp*. This deduction aligns with established morphology-based identification methods for fungal taxa (Table 1).

Table 1 Identification of Fungi

Organism	Morphology	Microscopic observation
Penicillium Spp.	Conidiophore arises vertically on substrate mycelium which is septate. Conidiophores are short and breached at the tip into finger like clusters of sterigmata.	

The results presented in our study build upon previous research conducted in our laboratory, where banana peel substrates were identified as the most suitable for invertase production via solid-state fermentation, yielding 75 U/ml invertase activity after 96 hours (Table 2 and Figure 1) **(Gebresemati, M., & Gebregergs, A., 2015)**. This finding aligns with previous studies that have reported enhanced production of invertase when banana peel substrates were supplemented with sucrose **(Gebresemati, M., & Gebregergs, A., 2015)**. The selection of appropriate support materials in solid-state fermentation is crucial for microbial growth and product formation, as these materials serve as a source of nutrients **(Trivedi *et al*., 2015)**. Our results further demonstrate the significant impact of incubation periods on invertase production by *Penicillium spp.* A lag phase was observed during the initial 24 hours of spore germination, with minimal enzyme synthesis. Subsequently, invertase activity peaked after four days of incubation, followed by a decline in activity until the sixth day. This pattern is consistent with findings by **Monsan and Combes (1984)**, highlighting the temporal dynamics of invertase production in *Penicillium spp.* Solid-state fermentation using banana peel substrates offers a promising avenue for enhanced invertase production, with implications for various industrial applications. Table 2 summarize the key findings regarding invertase production under different conditions, providing valuable insights for future research and industrial applications.

Table 2 Result of Invertase Production at Different Time Interval

Solid substrate	Incubationtime (h)	Invertase activity(U/ml)	Specific activity(U/mg)
Banana peel	72	42	0.19
	96	75	0.26
	120	55	0.24

Legends: h - hour, U – unit, ml – millilitre, mg – milligram

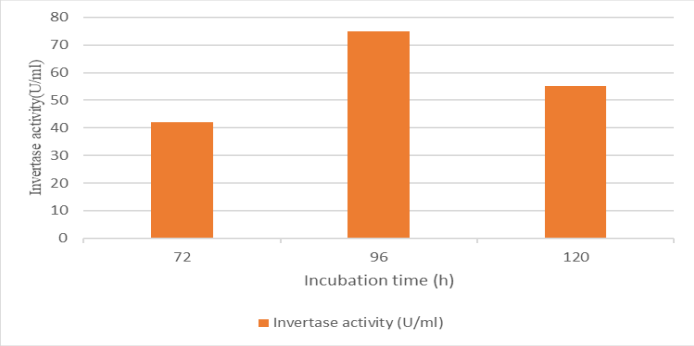


Figure 1 Invertase production at different time interval

Partial purification of invertase by ammoniumsulfate precipitation

Invertase, the enzyme responsible for producing oligosaccharides from sucrose, has been extensively studied for purification from various microbial and plant sources. In this investigation, conventional purification techniques were employed, including the use of ammonium sulfate fractionation. Ammonium sulfate fractionation involves salting out proteins by exposing them to high salt concentrations, a method widely utilized for protein fractionation by precipitation. This technique exploits the differential solubility of proteins at varying salt concentrations, with the precipitated protein typically retaining its activity upon redissolution. Moreover, these salts can confer stability against denaturation or proteolysis **(Chatterton *et al*., 1989)**. Initially, the crude invertase was subjected to 20% saturation with ammonium sulfate, resulting in a precipitate devoid of enzyme activity, which was subsequently discarded. The supernatant was further subjected to increasing levels of saturation (50%, 60%, 70%) with ammonium sulfate. Analysis of the resulting precipitate revealed a 3.19-fold purification of invertase, with a specific activity of 0.94 and a recovery rate of 11.66% (Table 3). These findings demonstrate the efficacy of ammonium sulfate fractionation in purifying invertase from the crude extract, paving the way for subsequent downstream applications and further characterization of the enzyme.

Invertase production using laboratory isolated *Penicillium spp.* under solid state fermentation

Table 3 Partial purification of invertase

Enzyme	Volume (ml)	Total protein (mg/ml)	Enzyme unit (U/ml)	Total Activity (U)	Specific Activity (U/ml)	Fold purification	% Yield
Crude	100	14	42	4200	3	1	100
Precipitation (70%)	10	52	49	490	0.94	3.19	11.66

Legend: % - percentage

Result of qualitative detection of glucose, fructose and sucrose

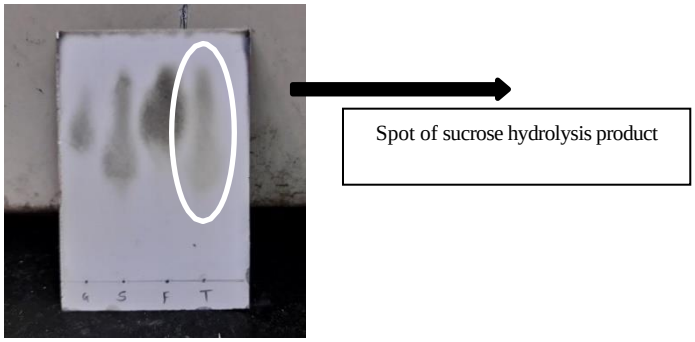


Figure 2 Presence of OS was confirmed by back color spot on TLC plate

Characterization of magnetic nanoparticles

Preliminary characterization

From microscopic analysis, the result stated that, magnetic nanoparticles and chitosan-coated magnetic nanoparticles differ in size and surface area, which is show in Figure 3 and further analysis was performed using a particles size analyzer.

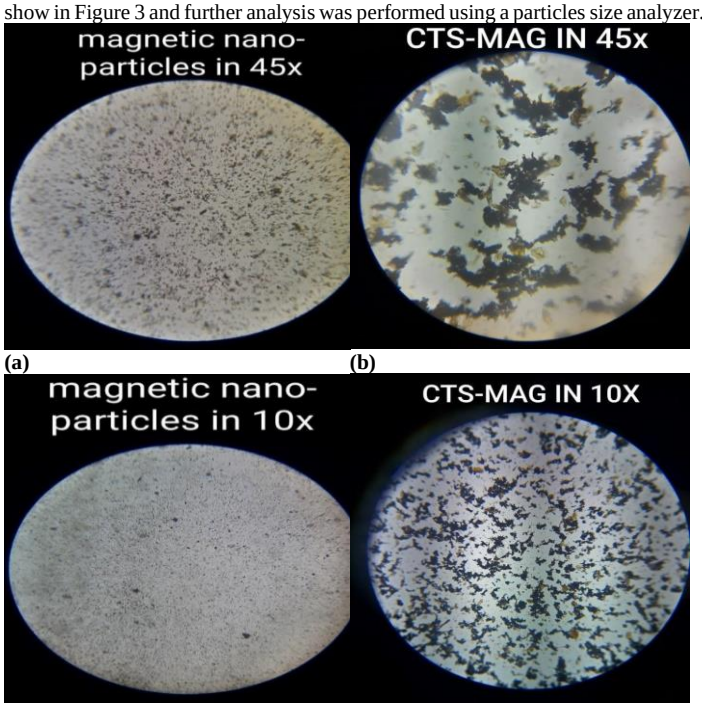


Figure 3 Microscope picture of (A)Magnetic nanoparticles in 10x (B) Magnetic nanoparticles In 45x (C) Chitosan magnetic nanoparticles in 10x (D) Chitosan magnetic nanoparticles in 45x

Separation of magnetic nanoparticles

The successful separation of enzyme-coated chitosan magnetic nanoparticles using a permanent magnet underscores the utility of magnetic separation techniques in nanoparticle applications. By exploiting the magnetic properties of the nanoparticles, this approach offers several advantages, including simplicity, efficiency, and the potential for scalability. Furthermore, the ability to easily separate the nanoparticles from the suspension is crucial for downstream applications, such as enzyme immobilization or catalysis. This straightforward separation method facilitates the purification of the enzyme-coated nanoparticles, ensuring their efficacy in various biotechnological and industrial processes. Overall, the successful separation of enzyme-coated chitosan magnetic nanoparticles using a permanent magnet highlights the potential of magnetic separation techniques for nanoparticle-based applications, offering a versatile and efficient means of particle extraction and purification.

Particle size distribution of magnetic nanoparticles

The size distribution histogram obtained from zeta potential measurements (Figure 4) and dynamic light scattering (DLS) analysis (Figure 5) collectively provide valuable insights into the characteristics of the magnetic nanoparticles synthesized in this study. The DLS histogram indicates a size range for the nanoparticles spanning from 100 to 1000 nm, suggesting a diverse population of particles with varying diameters. Additionally, the zeta potential measurement reveals a negative zeta potential value of -25.9 mV, indicating a moderate surface charge on the nanoparticles. These magnetic nanoparticles were synthesized through the co-precipitation method involving Fe^{2+} and Fe^{3+} ions, chitosan, and glutaraldehyde. The presence of several large particles observed in the size distribution histogram may be attributed to the agglomeration of smaller nanoparticles during synthesis or subsequent processing steps. Despite the presence of agglomerates, the overall size distribution suggests the successful formation of magnetic nanoparticles within the desired size range The combination of DLS and zeta potential measurements offers valuable information regarding the size and surface charge characteristics of the synthesized magnetic nanoparticles. Understanding these properties is crucial for optimizing their performance in various applications, including drug delivery, magnetic separation, and biomedical imaging. Further characterization and optimization efforts could focus on controlling particle size distribution and mitigating agglomeration to enhance the uniformity and stability of the magnetic nanoparticles for targeted applications.

Yield of enzyme immobilization

The yield of enzyme immobilization was assessed by measuring the remaining enzyme activity in the liquid phase after immobilization of invertase from *Penicillium spp.* onto chitosan granules activated by glutaraldehyde. The results indicated a yield of enzyme immobilization, expressed as YI, of approximately 59.01% (Table 4). These findings underscore the success of the immobilization method employed and the effective immobilization of invertase onto chitosan magnetic nanoparticles. The observed recovery of enzyme activity following immobilization is influenced by the properties of the carrier material, as it can affect the extent of enzyme distortion at various levels induced by immobilization. In this context, the activation of chitosan granules by glutaraldehyde played a crucial role. Glutaraldehyde activation significantly reduced the ability of the immobilized enzymes to desorb from the supports due to the high ionic strengths conferred by this activation method. This suggests that glutaraldehyde promoted a strong support-protein interaction, enhancing the stability of the immobilized enzyme. The reported approach offers a simple yet effective strategy for achieving excellent enzyme immobilization rates and desired stability, which holds promise for various biotechnological applications. These findings are consistent with previous studies emphasizing the importance of carrier materials and activation methods in optimizing enzyme immobilization efficiency and stability **(López-Gallego *et al.*, 2005)**. Overall, the results highlight the potential of the developed immobilization method for enhancing the performance and applicability of invertase enzymes in biotechnological processes.

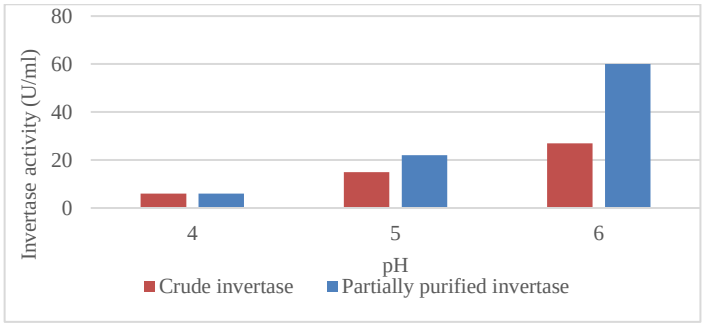
Table 4 invertase activity and % yield of immobilization

Immobilized enzyme/ filtrate	Invertase activity (U/ml)	% Yield of immobilization
Immobilization	25	59.01
Filtrate	61	

Result of characterization and stability of crude, partially purified and immobilized invertase

Effect of pH on invertase activity and stability

The effect of pH on the activity of crude invertase and partially purified enzyme was investigated across a pH range of 4-6. For crude invertase, the maximum activity of 27 U/ml was observed at pH 6, decreasing to 6 U/ml at pH 4. Similarly, the partially purified enzyme exhibited a maximum activity of 60 U/ml at pH 6, diminishing to 6 U/ml at pH 4 (Figure 6 (A)). Additionally, the stability of the crude invertase is 48 U/ml at pH 6 and decreased to 4 U/ml at pH 4. Similarly, the partially purified enzyme displayed a maximum stability of 52.9 U/ml at pH 6, declining to 6 U/ml at pH 4 (Figure 6 (B)). These findings underscore the influence of pH on invertase activity, with optimal activity observed at pH 6 for all enzyme forms. Additionally, the decrease in activity at lower pH values suggests a pH-dependent denaturation or inhibition of the enzyme. The stability data further indicate that the invertase enzymes maintain their activity most effectively at pH 6, highlighting the importance of maintaining appropriate pH conditions for enzyme function and stability in biotechnological applications. Overall, these results provide valuable insights into the pH-dependent behaviour of invertase enzymes and their potential utility in various industrial processes.



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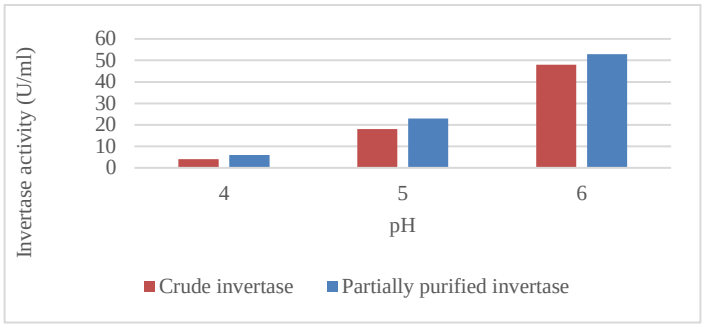
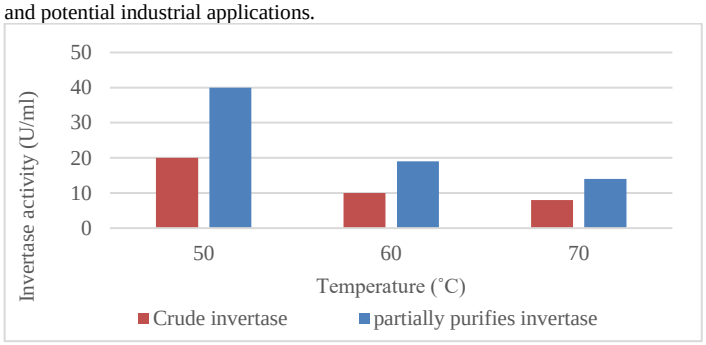


B

Figure 6 Effect of pH on (A) Invertase Activity and (B) Stability

Effect of temperature on invertase activity and stability

The effect of temperature on the activity of crude invertase and partially purified enzyme was assessed across a temperature range of 50-70 °C. For crude invertase, the maximum activity of 20 U/ml was observed at 50 °C, decreasing to 8 U/ml at 70°C. Similarly, the partially purified enzyme displayed a maximum activity of 40 U/ml at 50°C, declining to 14 U/ml at 70°C (Figure 7(A)). However, only optimum reaction temperature of immobilized enzyme was investigated in this study. Remarkably, the optimum reaction of the immobilized enzyme exhibited a significant increase compared to crude and partially purified invertase. At 70°C, the maximum invertase activity reached 77 U/ml, declining to 50 U/ml at 50°C for the immobilized enzyme (Figure 7(C)). Additionally, the stability of the crude invertase is 19.56 U/ml at 50°C and decreased to 9 U/ml at 70°C. Similarly, the partially purified enzyme displayed a maximum stability of 38 U/ml at 50°C, declining to 14 U/ml at 70°C (Figure 7(B)). These findings highlight the enhanced activity and stability of the immobilized enzyme compared to its crude and partially purified counterparts. The significant increase in activity at higher temperatures suggests that immobilization may confer thermal stability to the enzyme, making it suitable for applications requiring elevated temperatures. However, further characterization of the immobilized enzyme under different conditions could provide a more comprehensive understanding of its performance and potential industrial applications.



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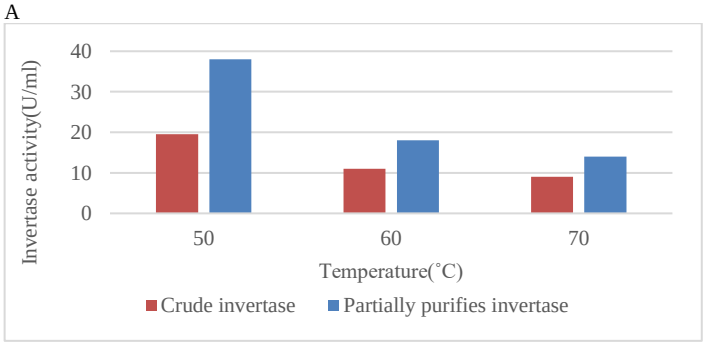

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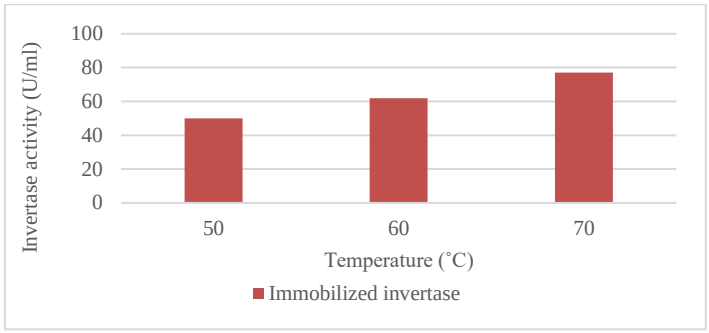
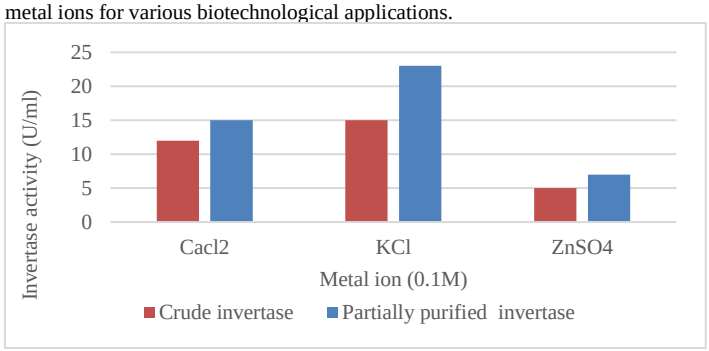


C

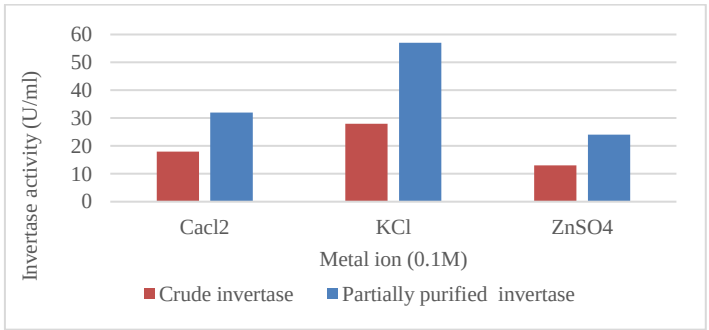
Figure 7 Effect of temperature on Invertase activity (A) Stability (B) and Immobilized invertase (C)

Effect of different metal ions on invertase activity

The effect of different metal ions on the activity of crude invertase and partially purified enzyme was investigated, with a focus on potassium chloride (KCl) and zinc sulfate ($ZnSO_4$) at a concentration of 0.1M. For crude invertase, the maximum activity of 28 U/ml was recorded in the presence of 0.1M KCl, which decreased to 13 U/ml in the presence of 0.1M $ZnSO_4$. Similarly, the partially purified enzyme exhibited a maximum activity of 28 U/ml in 0.1M KCl, decreasing slightly to 13 U/ml in 0.1M $ZnSO_4$ (Figure 8(A)). For crude invertase, the maximum stability of 15 U/ml was recorded in the presence of 0.1M KCl, which decreased to 5 U/ml in the presence of 0.1M $ZnSO_4$. Similarly, the partially purified enzyme exhibited a maximum activity of 23 U/ml in 0.1M KCl, decreasing slightly to 7 U/ml in 0.1M $ZnSO_4$ (Figure 8(B)). These results suggest that the activity of invertase is influenced by the presence of specific metal ions, with KCl showing a more favourable effect compared to $ZnSO_4$. Additionally, while the stability of invertase activity appears to be maintained in the presence of KCl, immobilized invertase did not exhibit activity in the presence of various metal ions, indicating a potential limitation of the immobilization process under these conditions. Further investigation may be warranted to elucidate the underlying mechanisms and optimize the performance of immobilized invertase in the presence of different metal ions for various biotechnological applications.



A



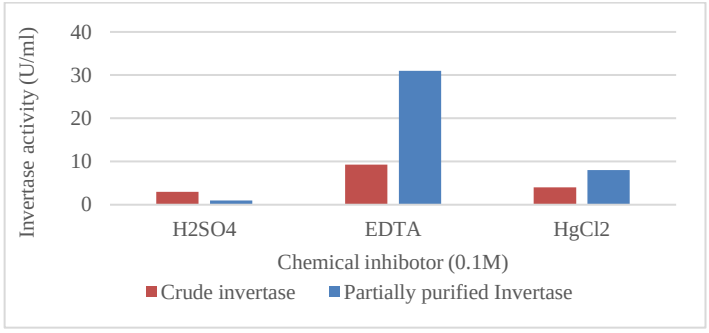
B

Figure 8 Effect of metal ion on Invertase activity (A) and Stability (B)

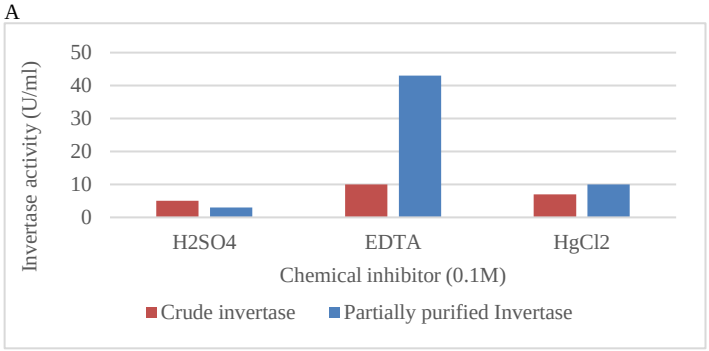
The effect of different chemical inhibitors on invertase activity and stability

The effect of different chemical inhibitors on the activity of crude invertase and partially purified invertase was evaluated by pre-incubating the enzymes at 30°C for 30 minutes with various inhibitor concentrations before conducting the standard invertase activity assay with sucrose. For crude invertase, the maximum activity of 10 U/ml was recorded in the presence of 0.1M EDTA, which decreased to 5 U/ml in the presence of 0.1M H_2SO_4. Similarly, the partially purified enzyme exhibited a maximum activity of 43 U/ml in 0.1M EDTA, decreasing to 3 U/ml in 0.1M H_2SO_4 (Figure 9(A)). Moreover, the stability of crude invertase reported maximum stability of 9.31 U/ml was recorded in the presence of 0.1M EDTA,

which decreased to 3 U/ml in the presence of 0.1M H_2SO_4. Similarly, the partially purified enzyme exhibited a maximum stability of 31 U/ml in 0.1M EDTA, decreasing to 1 U/ml in 0.1M H_2SO_4 (Figure 9(B)). These results indicate that the activity of invertase is affected by the presence of specific chemical inhibitors, with EDTA showing a more favourable effect compared to H_2SO_4. Additionally, while the stability of invertase activity appears to be maintained in the presence of EDTA.



A



B

Figure 9 Effect of chemical inhibitor on Invertase activity (A) and Stability (B)

Application of immobilized invertase by developing a continuous bioconversion system for invert syrup production.

In a continuous system, 1 ml of 5% sucrose solution prepared in 0.6 ml of 0.1M phosphate buffer (pH 5) was fed from the reservoir at an optimal flow rate of 0.82 ml/h. The continuous system was maintained in a water bath at a temperature of 50°C. This setup represents a continuous enzymatic process where the substrate (sucrose solution) is continuously supplied to the system at a controlled flow rate (Figure 10). The enzymatic conversion of sucrose into invert syrup is facilitated by the presence of invertase enzyme, which exhibits its optimal activity at the specified conditions of pH 5 and 50°C. The resulting invert syrup contains the enzymatically converted products of sucrose, primarily glucose and fructose, indicating the efficiency of the continuous enzymatic process. Overall, this continuous enzymatic system demonstrates the potential for large-scale production of invert syrup through the enzymatic hydrolysis of sucrose, offering advantages such as precise control over reaction conditions, high product yield, and continuous operation.

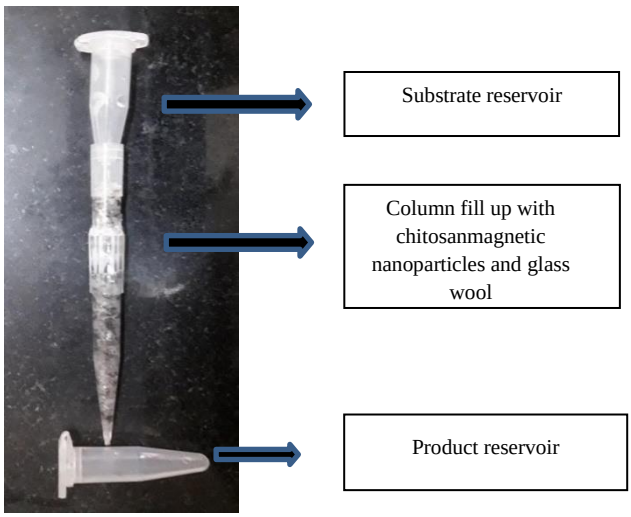


Figure 10 System of continuous bioconversion for invert syrup production

CONCLUSION

Finally, we conclude that *Penicillium spp.* potentially produces invertase and yields high amounts on banana peels through solid-state fermentation, utilizing fruit waste material. The crude and partially purified invertase activity was observed to be at its maximum at pH 6 and 50 °C, in the presence of potassium chloride (a metal ion) and EDTA (a chemical inhibitor). Successful immobilization of invertase was achieved through covalent binding onto chitosan-coated magnetic nanoparticles, resulting in a 59.01% immobilized yield. When using chitosan-coated magnetic nanoparticles, the optimum reaction temperature range was found to be from 50 to 70 °C, with the highest reaction observed at 77°C. Moreover, qualitative analysis via thin-layer chromatography confirmed the enzymatic hydrolysis of sucrose into its constituent monosaccharides, glucose, and fructose, validating the industrial potential of the *Penicillium spp.* derived invertase. A continuous bioconversion system proved successful in producing invert syrup.

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