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Isolation of Fructosyl Transferase from *Aspergillus niger* Strain SS274 and Enhancing its Stability by Harnessing Magnetic Nanoparticles Assisted Enzyme Immobilization Strategy

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Key words
FTase, Fructooligosaccharides, Prebiotics, HPLC, Magnetic nanoparticles, FTIR

ABSTRACT

Fructosyl trasferase is considered as an important industrial enzyme for production of prebiotics that hold numerous health benefits, is purified from *Aspergillus niger* strain SS274 using size exclusion chromatography Sephadex G-100 showed 91% of sample recovery, 3.57 purification fold and 393.32U/mg of specific activity. It was a glycoprotein dimer enzyme of approximately 75kDa molecular weight with 2 subunits of 45kDa and 30kDa which showed optimal activity from 50-60 ℃ and at pH-5 and its activity was increased up to 4% from normal activity in presence of Fe metal ions while Hg and Ag showed inhibitory effect on enzyme's activity. Formation of prebiotics or fructooligosaccharides (FOS) was confirmed through HPLC analysis using sucrose as a substrate. Enzyme was further immobilized on magnetic nanoparticles using carbodiimide method to make it more industrial viable by increasing it shelf life and stability, its activity was observed in mobilize and immobilize state for period of 6 months stored at 4 ℃ and -20 ℃ and it showed maximum activity and better shelf life at -20 ℃ in immobilize form. Fructosyl transferase from *A. niger* have good potential for FOS production at industrial scale for health applications.

INTRODUCTION

Nowadays, foods that contributes to maintain good health have gained prominence due to their increased demand. People are getting more conscious about their food intake and possible effect of the ingredients present in food (Trujillo *et al*., 2019). Non-digestible ooligosaccharides are one among of them due to their health-related benefits among consumers (Mussatto and Mancilha, 2007). Fructooligosaccharides (FOS) belong to the class of oligosaccharides, that are low molecular weight carbohydrates, which basically contained 2 to 10 monosaccharides units linked through glycosidic linkages (Sabater-Molina *et al*., 2009). FOS being low caloric, mild sweet and non-carcinogenic are one of the important ooligosaccharides (Dhake and Patil, 2007; Pool-Zobel *et al*., 2002) and have proved prime in proliferating gut microflora of humans (Antošová and Polakovič, 2001; Campbell *et al*., 1997; Ozcan and Kurtuldu, 2014). They could easily be fermented by gut microflora bifidobacterial and lactobacilli to enhance their growth and function (Markowiak and Ślizewska, 2017; Trujillo *et al*., 2019). FOS due to their lower molecular weight show hygroscopic function and play important role in water retention, they also show good thermal stability due to their higher viscosity (Crittenden and Playne, 1996; Roberfroid, 2007). FOS possess many health benefits, cholesterol lowering effect, mineral absorption in body and cancer suppression has been studied throughout many years (Khanvilkar and Arya, 2015; Pool-Zobel *et al*., 2002; Rao, 2001).

FOS are represented by formula GF_n, chain of fructose oligomers attached with terminal glucosyl (Michel *et al*., 2016; Sangeetha, 2003). 1-kestose (GF_2), 1-nystose (GF_3) and 1-β-fructofuranosylnystose (GF_4) are frequent FOS used in functional foods (Borromei *et al*., 2009; Dake and Kumar, 2012).Chemical structure of FOS is given

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in Figure 1 Naturally FOS are present in many fruits and vegetables like banana, onion, garlic chicory, barley and oats, but in low concentrations that they cannot carry out their health-related functions properly (Sridevi *et al*., 2014; Yun, 1996).

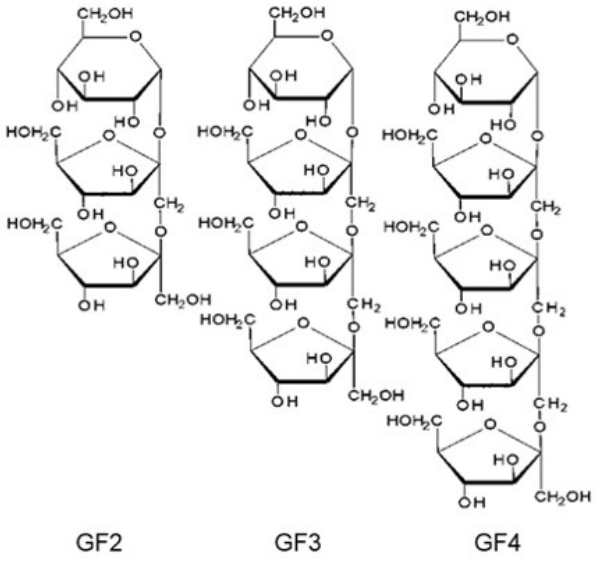

Fig. 1. Chemical structure of 1-kestose, 1-nystose and 1-β-fructofuranosyl nystose.

FOS can be enzymatically produced by the action of Fructosyltransferases or (Ftase) enzymes [EC 2.4.1.9] which carry out the transfructsylation of sugars like sucrose, glucose, maltose and fructose (Sangeetha *et al*., 2004; Yang *et al*., 2016). Many microorganisms and plants could be source of Ftases (Arthee and Vijila, 2014; Cunha *et al*., 2019; Dake and Kumar, 2012; Fujishima *et al*., 2005; Heyer and Wendenburg, 2001; Itaya *et al*., 1999; Kingston Smith *et al*., 1999; Koops and Jonker, 1996; Luscher et al., 1993; Lasseur et al., 2009; Van Hijum *et al*., 2002). FTase catalyzes the chain of reaction employing disproportionate mechanism in which fructosyl moiety of donor sucrose is transferred to another sucrose molecule to release glucose and ooligosaccharides and so on, mechanism can be shown as follows:

$$GF + GF_n \rightarrow G + GF_{n+1}$$

$$GF + GF_{n+1} \rightarrow G + GF_{n+2}$$

FOS being non-digestible fibers, play an important role in cancer suppression by increasing short chain fatty acids (SCFA) like propionic and butyric acid in gut gradually increasing the proliferation of gut microflora. Increasing SCFA in gut will ultimately decrease the fecal pH and bile acids that are main contributors of colorectal cancer (Bruno-Barcena and Azcarate-Peril, 2015; Dou *et al*., 2022; Grajek *et al*., 2005; Pool-Zobel *et al*., 2002; Rao, 2001).

This study aims to purify extracellular Ftase from *Aspergillus niger* SS274, its biochemical characterization for production of FOS and its immobilization on MNPs to enhance its shelf life.

MATERIALS AND METHODS

Aspergillus niger SS274 pure culture was obtained from AMBER laboratory of School of Biochemistry and Biotechnology, University of the Punjab, Lahore used as an inoculum for pre-inoculum media. A loop full of stored inoculum was transferred to pre-inoculum media (yeast extract: 0.2%, sucrose: 2%, pH: 6) under sterile conditions and incubated for 24 h at 30℃ at 100 rpm.

Fermentation media

For extracellular enzyme production 500ml of fermentation media (Sucrose: 4%, Yeast extract: 10g/L, $NaNO_3$: 1g/L, KH_2PO_4: 1g/L, K_2HPO_4: 0.5g/L, NaCl: 0.25g/L, $MgSO_4.7H_2O$: 0.05g/L, NH_4Cl: 0.05g/L, pH-6) was prepared and inoculated with 24h old pre-inoculum media (20%). Fermentation media was incubated at 30℃ for 72 h at 200 rpm (Arthee and Vijila, 2014; Ojwach *et al*., 2020). Fermentation media without inoculum was used as a control and all reaction were run in duplicates.

Preparation of extracellular enzyme extract

After incubation fermentation media was filtered through Whatman No.1 filter paper and filtrate broth was centrifuged at 4℃ for 20 min at 8000 rpm, supernatant was separated and served as crude mycelial enzyme extract. Mycelial extract was subjected to different ammonium sulphate concentrations (20%, 30%, 40%. 50%, 60%,70% and 80%), respectively. The precipitate was obtained by centrifugation at 4℃ for 20 min at 13,000 rpm. Pellets were dissolved in 0.2M phosphate buffer saline pH-7.5 and dialyzed further by employing 15KDa cut off dialyzing tubing to remove any ammonium sulphate left behind. After dialysis extracellular FTase was further purified through size exclusion chromatography using Sephadex G-100 (1.5× 30cm) column, equilibrated twice with elution buffer (50mM Tris-Cl, 150mM NaCl and 10% Glycerol, pH: 8, using flow rate of 0.5ml/min. Each fraction was analyzed for FTase activity. Concentration of protein was determined by Bradford reagent. Extracellular FTase was used to perform enzyme assays and biochemical analysis.

Enzyme assay

Enzyme activity of extracellular Ftase is defined as µmol of glucose release during enzymatic reaction

per minute under defined standard conditions. Enzyme activity was measured by DNS (Dinitrosalicylic) assay. For enzymatic reaction sucrose was used as a substrate, 20% of sucrose solution was prepared in 0.1M citrate buffer of pH-5. 1ml extracellular enzyme solution and 1ml of substrate solution were mixed together and placed in water bath at 55℃ for 60 min at 120 rpm. At the end of specified incubation time, reaction mixture was placed in boiling water bath for 5-10 min to stop enzymatic reaction. Reaction mixture was cooled at room temperature and centrifuged at 10,000 rpm for 5 min at 4℃. 2ml of DNS reagent was added in reaction mixture and boiled for 10 min. Absorbance of reaction mixture was measured at 540nm along control sample.

Optimal temperature, pH, metal ion and carbon source

Enzyme activity was accessed on different temperature ranges (30℃-90℃) to determine optimal and maximum activity of enzyme. Enzymatic reactions were incubated at different temperatures, pH-5.5 for 60 min, while using substrate concentration 580mM. Enzyme activity was accessed on different pH ranges. Different buffer systems (0.1M Citrate buffers pH 3-6, 0.1M Phosphate buffer pH 7-8 and 0.1M Tris-HCl buffer pH 9-12) were employed to determine optimal enzyme activity, while other reaction conditions (Temperature:55℃ and time 60 min) were same. Before enzymatic reactions, enzyme was first incubated with all three buffer systems for 20 min at room temperature.

Effect of metal ions on Ftase activity was analyzed. 0.5M solutions of following chemicals were prepared: NaCl, $MgSO_4$. $7H_2O$, $HgCl_2$, $FeSO_4$, $ZnSO_4$, KCl, $CaCl_2$, $CuSO_4$ and AgCl. Before enzymatic reactions, 500µl of enzyme solution was incubated with 500µl of metal ion solution for each reaction and enzyme activity was measured. Substrate solutions of different concentrations (10%: 0.29M, 20%: 0.58M, 30%: 0.88M, 40%: 1.17M, 50%: 1.46M, 60%: 1.75M, 70%: 2.04M and 80%: 2.33M) sucrose substrate solutions of different concentrations were prepared in 0.1M citrate buffer of pH 5.5. An enzyme activity was analyzed for each substrate concentration at temperature 55℃ for 60 min. Effect of carbon source was also analyzed on the production of enzyme by using different carbon source in fermentation media. Fermentation media was prepared in same way mentioned earlier but sucrose is replaced with other potential carbon source maltose, fructose and glucose. Incubation was carried at 30℃ for 72 h at 200 rpm.

HPLC analysis

To confirm formation of FOS, HPLC analysis of enzymatic reaction and of fermentation broth was performed using amino column. Isocratic analysis was carried at room temperature with water and acetonitrile (30:70) as a mobile phase. Fow rate was adjusted to 1ml/min with wavelength 195nm. The yield of FOS was measured as percentage of conversion yield from starting substrate concentration. Analysis was done on basis sequence of retention time of 1-Kestose (GF2), 1-Nystose (GF3) and 1-fructofuranosyl nystose (GF4), respectively.

Synthesis and characterization of MNPs

Magnetic nanoparticles were synthesized by employing co-precipitation method (Samra *et al*., 2012) , 0.25mM solutions of $FeCl_2$ and $FeCl_3$ were mixed in ratio 1:2 and pH was adjusted to 10 with NH_4OH (30%). Resulting black solution was heated at 80℃ with continuous stirring until black precipitates were formed. Precipitates were firstly washed twice with deionized water secondly with acetone and later on dried under vacuum (70℃). 100mg of dried particles were suspended in 50ml deionized water and sonicated for 60 min, particles were filtered through 0.45µm filter. MNPs were characterized through FTIR (Fourier transform infrared), zeta potential, DLS (Dynamic light scattering) and SEM (Scanning electron microscope).

Conjugation of FTase with MNPs

Extracellular FTase was conjugated with MNPs through carbodiimide method. 50mg of MNPs were suspended in 1ml of deionized water and pH:8 was adjusted with NaOH (1M). 20mg of EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide) mixed in 1ml of deionized water and pH:6.4 was adjusted with HCL(1N). Both solutions were mixed at 120 rpm for 40 min, after 40 min 10mg/ml of EDC was added in solution. 0.2 mg of FTase was added in solution and incubated for 3 h in dark at 120 rpm. Particles were separated by centrifugation at 13,000 rpm for 15 min at 4℃ and washed twice with deionized water. Conjugation was confirmed by FTIR, particles were divided in different fractions and stored at both 4℃ and -20℃ for further analysis. Activity of extracellular FTase enzyme was analyzed in free and immobilized form for the period of six months stored at 4℃ and -20℃.

RESULTS AND DISCUSSION

Aspergillus niger SS274 was analyzed for the production of Ftase during submerged fermentation. The crude enzyme extract was obtained after the end of incubation following filtration and centrifugation. Activity of extracellular FTase enzyme was analyzed through DNS method. The crude enzyme extract was subjected

to different, from 20% to 80% ammonium sulphate precipitation. 30% of precipitate gave ideal concentration of ammonium sulphate for FTase precipitation showing FTase activity. Dialysis was performed to remove extra ammonium sulphate and gave 0.186mg/ml enzyme hence increasing the purity to 2.28-fold. Specific activity of FTase was increased from 88.69U/mg to 393.32U/mg after size exclusion purification. Detailed comparison of purification of FTase is given in Table I. Purified FTase is of 75kDa, 2 subunits of 45kDa and 30kDa are responsible for activity of FTase obtained after SEC shown in Figure 2.

Table I. Comparison of different purification steps on Specific Activity of extracellular FTase.

Steps	Enzyme activity (µmol/min)	Enzyme (mg)	Specific activity U/mg	Recovery of sample %	Purification fold
Enzyme extract	67.76	0.764	88.69	100.00	1.00
Ammonium sulphate	41.85	0.38	110.14	61.77	1.24
After dialysis	37.54	0.186	201.80	55.40	2.28
Size exclusion	38.15	0.097	393.32	91.16	3.57

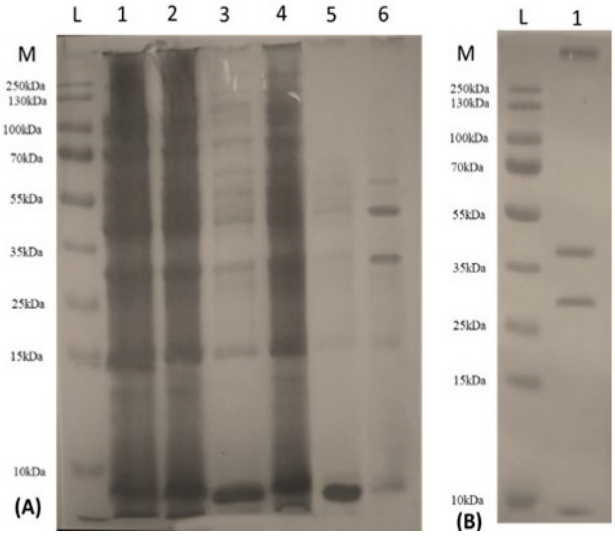

Fig. 2. Extracellular FTase purification fractions of each step run on 12% SDS-PAGE. (A) Lane L: pre-stained protein ladder, Lane 1, 2: Protein bands after 70% and 60% Ammonium Sulphate precipitation; Lane 3,4: Protein bands after 40% and 50% Ammonium Sulphate precipitation; Lane 5, 6: Protein bands after 30% Ammonium Sulphate precipitation and after Dialysis and freeze drying. (B) Two subunits of extracellular FTase (45kDa and 30kDa) obtained as a result of SEC.

Biochemical analysis of extracellular purified enzyme was performed to determine temperature, pH, substrate concentration and metal ion effect on FTase. Effect of temperature was studied to determine optimum temperature for enzyme's activity, which was subjected to different temperature ranges 30°C to 90°C shown in Figure 3A.

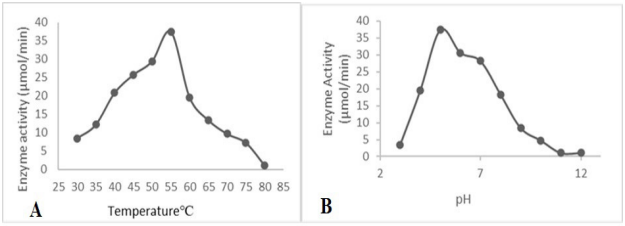

Fig. 3. (A) Effect of temperature (A) and pH (B) on activity of extracellular FTase.

FTase gave its maximum activity at 55°C, while enzyme was still showing 70% activity from 40°C to 60°C. FTase retained its 40% activity at 30°C and 65°C. Enzyme's performance was recorded lowest only 5% at 80°C and no activity was recorded at 90°C at all. Effect of pH was recorded by subjecting FTase to different pH ranges (3-12) from acidic to basic using different buffer systems shown in Figure 3B. FTase showed its maximum activity at pH 5. FTase retained its 80% activity from pH 6-7 and maintained 50% activity at pH 4 and pH 8. Activity of FTase started to decline from pH 9 and almost no activity was recorded at pH 12. 0.5mM solution of different metal ions Na^{+}, Ca^{2+}, Mg^{2+}, K^{+}, Fe^{2+}, Hg^{2+}, Zn^{2+}, Cu^{2+} and Ag^{2+} were prepared and FTase activity was recorded in the presence of these metal ions. Residual activity of FTase in presence of each metal ion is shown in Table II.

Table II. Effect of metal ions on residual activity of FTase.

Metal ions	Concentration	Residual activity %
Control	0mM	100
Mg^{2+}	0.5mM	83.53
Na^{+}	0.5mM	90.12
K^{+}	0.5mM	93.41
Fe^{2+}	0.5mM	104.94
Ag^{2+}	0.5mM	15.35
Ca^{2+}	0.5mM	96.71
Zn^{2+}	0.5mM	95.39
Cu^{2+}	0.5mM	81.56
Hg^{2+}	0.5mM	13.70

Activity of FTase without any metal ion was taken as 100% or control. Out of all metal ions ferrous enhanced the enzyme residual activity to 4% when compared with its original activity. Ca^{2+}, Zn^{2+}, Na^{2+} and K^{+} had slightest effect on enzyme's activity, decreasing its activity 5-7% while Mg^{2+} and Cu^{2+} caused decrease in FTase activity from 15-20%. Ag^{2+} and Hg^{2+} had decreased the enzyme activity up to 85% indicating these metal ions has shown inhibitory effect on activity of enzyme. Out of all Ferrous could be a better choice to enhance the activity of FTase for better production of FOS.

FTase production was also analyzed in the presence of different carbon sources (sucrose, glucose, maltose and fructose). Sucrose and glucose were proved better carbon source for FTase production shown in Figure 4.

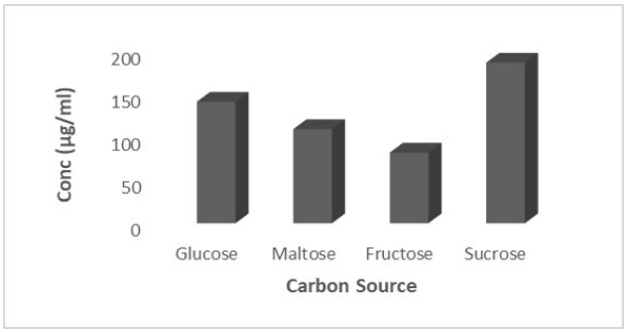

Fig. 4. Effect of different carbon sources on extracellular FTase production.

FTase activity was calculated with increasing substrate concentration, with increasing substrate concentration activity of the enzyme gradually increase up to 70% of the substrate, after that enzyme activity ceased to increase showing enzyme is fully saturated. K_m and V_{max} of FTase was calculated by Michaelis Menten Kinetics equation by using enzyme activity and substrate relation. K_m and V_{max} were recorded 321mM and 64µM/min respectively. FOS production was confirmed through HPLC shown in Figure 5 and Table III.

To increase the stability and shelf life of FTase, it was immobilized on MNPs which were synthesized by co-precipitation method and characterized. Characterization of MNPs is shown in Figure 6, shows formation of amine functionalized MNPs of around 20-60nm in size were formed with circular morphology and stable while suspended in solution. Conjugated MNPs with FTase were characterized by FTIR, stretching peak at 1637.2cm^{-1} confirmed the formation of FTase immobilization with formation of amide bond. Schematic representation of MNPs synthesis and FTase conjugation is shown in Figure 7.

Table III. HPLC detail of products formed during FTase catalyzed reaction and in fermentation broth.

Products	FTase reaction		Fermentation broth	
	Peak area	Area %	Peak area	Area %
Sucrose	45074706	68.566	23311507	48.176
Glucose	3454595	5.255	6943798	14.350
Fructose	3242312	4.932	5119657	10.58
1-Kestose	8324399	12.663	3121757	6.452
1-Nystose	3076288	4.680	121177	0.250
1-Fructofura-nosyl nystose	1367211	2.080	23980	0.050

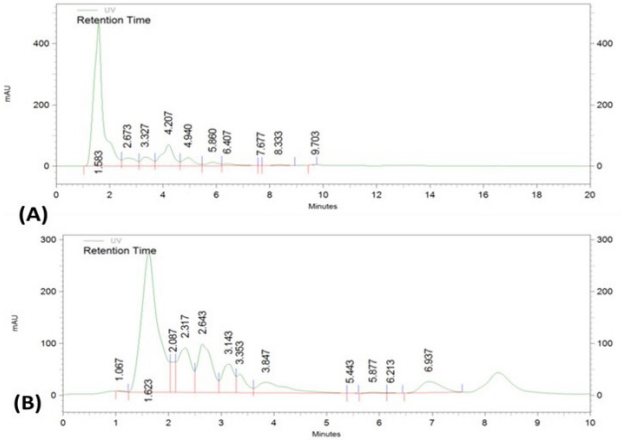

Fig. 5. HPLC chromatogram of FTase reaction (A) showing1-Kestose (GF2), 1-Nystose (GF3) and 1-Fructofuranosyl nystose (GF4). HPLC chromatogram of Fermentation broth (B) showing 1-Kestose (GF2), 1-Nystose (GF3) and 1-Fructofuranosyl nystose (GF4).

Immobilize and free state FTase were stored at two different temperatures (4°C and -20°C) for period of 6 months to study better storage condition and shelf life of FTase by analyzing enzyme's activity shown in Figure 8. FTase activity at first month was considered as 100% and compared with respective months accordingly. At 2nd month activity of FTase in free state stored at 4 °C decreased to 47.19% and at 3rd month it showed further decline of 12.86% and it continued to decrease to 1.41% in 4th month while no activity was observed at 5th and 6th month, respectively. Free state FTase activity stored at -20 °C was recorded 91.93% at 2nd month, 80.60% at 3rd month, 74.13% at 4th month, 54.73% at 5th month while 35.33% at the end of 6th month

FTase conjugated on MNPs were evaluated for its stability, at 2nd month MNP conjugated FTase stored at 4 °C showed 89.40% activity, it declined to 51.93% in

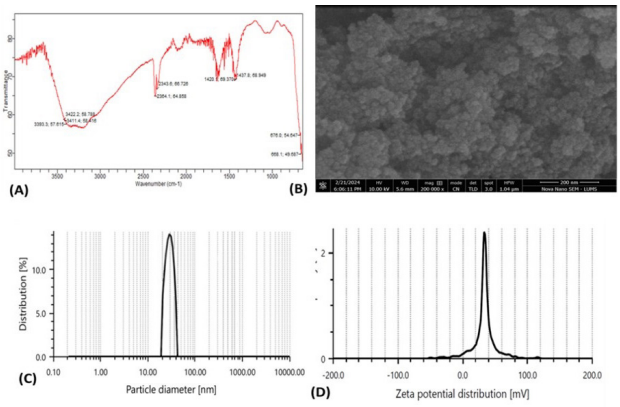

Fig. 6. Characterization of synthesized MNPs. (A) FTIR analysis of MNPs, stretching peak at 1420cm-1 and 1437cm-1 showed amine functionalized MNPs. (B) SEM analysis of MNPs at 200nm scale showed particles of circular morphology with almost 20-50nm in size. (C) DLS profile of MNPs showed particle of 30-60nm ± 10nm in size and its stability in solution while suspended. (D) Zeta potential of MNPs were recorded at 33.1±1.2mV with distribution peak of 33.5Mv.

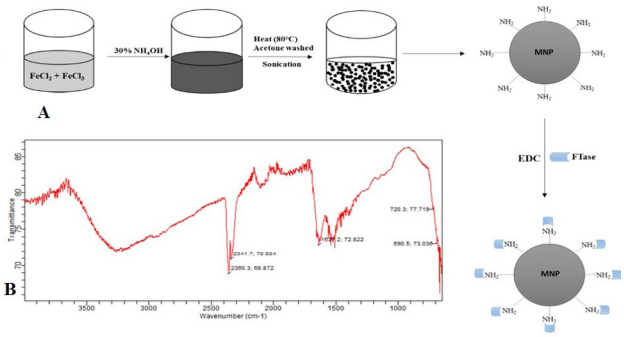

Fig. 7. (A) Schematic diagram of MNPs synthesis and their conjugation with FTase through carbodiimide method (B) FTIR analysis of FTase conjugated MNPs, stretching peak at 1637.2cm-1 indicates the formation of amide bond between MNPs and FTase enzyme.

third month, 34.01% in 4th month, 14.43% in 5th month and to 4.65% in 6th month. In the same way activity of MNP conjugated FTase stored at -20 ℃ was recorded which showed 95.35% of activity at 2nd month, 89.43% at 3rd month, 81.44% at 4th month, 75.03% at 5th month and 65.42% at 6th month. These results showed FTase showed higher stability and better shelf life in immobilize form and -20 ℃ is optimum storage temperature for FTase to sustain its maximum activity. FTase from different sources and organisms exhibit different activity and catalytic properties which has increased importance of its purification and analysis from different sources for industrial production of prebiotics, this study showed purified FTase enzyme with excellent potential of prebiotic production on industrial scale especially when immobilized on MNPs for enhanced stability as well as activity.

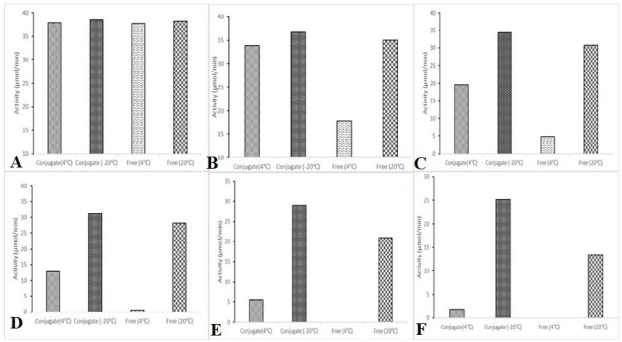

Fig. 8. Activity of Extracellular FTase for both, conjugated on MNPs and as free Enzyme for period of 6 months at 4 ℃ and -20 ℃. (A) FTase activity measured at 1st month with higher activity (B) FTase activity measured at 2nd month, significant decline in activity was measured at 4 ℃, while major decline was observed in free state (C) FTase activity at 3rd month, again activity show significant decline at 4 ℃ in free state (D) FTase activity measured at 4th month, major decline in enzyme's activity was observed at 4 ℃ in both conjugated and significantly in free state (E) FTase activity measured at 5th month, again more decline was observed at 4 ℃ for enzyme at conjugated form while no activity was observed for enzyme preserved in free state (F) FTase activity measured at 6th month, enzyme showed little activity in conjugated form stored at 4℃ while no activity was observed in free state, meanwhile a little decline was observed in FTase activity at -20 ℃ especially for enzyme conjugated on MNPs.

CONCLUSION

Extracellular FTase was successfully isolated and purified from *A. niger* SS274 that can be employed for prebiotic production and its was successfully immobilized to MNPs in order to increase its stability as well as shelf life making it more suitable for industrial applications.

DECLARATIONS

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IRB approval

The punjab University Ethics Review Board reviewed and approved the study (No. D/171/FIMS, Sated: 12/10/2023).

Generative AI and AI-assisted technology statement

The authors have declared that no generative AI or AI-assisted technologies were used to create this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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