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PARTIAL PURIFICATION AND CHARACTERIZATION OF FRUCTOSYLTRANSFERASE FROM *AUREOBASIDIUM PULLULANS*

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Abstract: Fructosyltransferase enzymes was isolated and partially purified from *Aureobasidium pullulans.* The enzyme being intracellular was recovered from the fungal sp. by extraction method using citrate buffer (0.1 M; pH 5.5). Enzyme was Semipurified by salt fractionation method which results increase in specific activity of the enzyme. Study regarding kinetic behavior indicates that the enzyme exhibits maximal activity at pH 5.5 and at the temperature of 50°C. Mn^{+2} & Mg^{+2} are activators of enzyme while Hg^{2+} is a strong inhibitor of enzyme activity. Reaction mixture resulting from enzyme action on sucrose exhibits presence of glucose, fructose and some short chain fructooligosaccharides.
Key words: fructosyltransferase, fructans, levans, glycosyltransferase, Aureobasidium pullulans.

1. Introduction:

Fructosyltransferases (FTase) catalyze the transfer of a fructose unit from one sucrose/fructan to another and are engaged in the production of fructooligosaccharide(FOS)/fructan. The enzyme is named **inulosucrase** (EC 2.4.1.9) when it synthesizes inulin-type fructans, and **levansucrase** (EC 2.4.1.10) when the polymerization product is levan. This enzyme belongs to the family of glycosyltransferases, specifically the hexosyltransferases.

Fructosyltransferase (FTF) enzymes produce fructose polymers (fructans) from sucrose. FOS have also been commercially produced using fructosyltransferases [Fructosyltransferase, EC 2.4.1.9] obtained from various microorganisms such as

Aspergillus foetidus, *Bacillus subtilis*, *Bacillus macerans*, *Streptococcus salivarius* and *Aureobasidium pullulans*. Fructosyltransferase can be obtained from a wide variety of sources such as microorganisms including bacteria and fungi (*Aureobasidium pullulans, Aspergillus niger, Aspergillus oryzae*, etc.) [1-4], higher plants asparagus, chicory, onion, Jerusalem artichoke, etc.) and animals. It has also reported that the FTFase activity is involved in a variety of processes: survival of bacteria in soil (*Bacillus subtilis*), phytopathogenesis (*Erwinia* and *Pseudomonas*) or symbiosis (*Bacillus polymixa*) for plant interactive bacteria [5]. In recent years, researchers

All bacterial fructosyltransferases catalyze the transfer of the fructosyl residue from the donor substrate i.e. sucrose to a variety of acceptor substrates, such as sucrose (kestose synthesis), fructan (fructan polymerization), glucose (sucrose synthesis), and fructose (bifructose synthesis). These enzymes utilize sucrose as the sole energy source for oligosaccharide synthesis (6). Fructosyltransferase also termed as levansucrase catalyze other transfrucolylation reactions e.g. the enzyme catalyzed formation of alkylfructosides from sucrose in the presence of alcohol [7, 8]. Fructosyltransferases are crucial for plant survival under stress conditions in species in which fructans represent the major form of reserve carbohydrate, such as perennial ryegrass (Lolium perenne).

Fructosyltransferase which catalyze the formation of plant fructans have wide scale applications as emulsifiers, sweeteners, control calcium balance and contribute to the reduction of antibiotic consumption [9] or a lowering of the concentrations of triglycerides, cholesterol, and insulin. FOS are fructose oligomers with a terminal glucose group, in which two-four fructosyl moieties are linked via α(1_2)-glycosidic bonds. Fructooligosaccharides (FOS) from sucrose are the new alternative sweeteners and possess a number of desirable characteristics such as low caloric, non-cariogenic, safety for diabetics, source of energy and acting as a growth factor for beneficial microorganisms in the intestinal flora.

In our work the source of enzyme used is *Aurteobasidium pullulans*, a dimorphic fungus. *Aureobasidium pullulans* is a saprophytic fungus that has a widespread occurrence. It commonly occurs on the surfaces of fruits and many other crop plants. It exhibits polymorphism in its life cycle which can grow as a budding yeast or mycelium depending upon environmental conditions. It is an industrially

important mold that has capacity to produce a polysaccharide termed 'pullulan' which is used commercially as a food ingredient as well as for coating and wrapping purposes. The present work involves isolation and partial purification of fructosyl transferase from *Aureobasidium pullulans* as well as studying its kinetic behavior.

Materials & Methods:

Pure culture of *A.pullulans (*NCIM-1048) *was* obtained from National chemical laboratory (NCL). It was maintained on the potato dextrose agar slants having pH 5.5

All the chemicals were purchased from Hi-Media. Glucose oxidase peroxidase kit was from Bio-Lab [10]. Glucose was estimated by glucose oxidase-peroxidase method. Protein was analysed by Lowry method [11].

Propagation of *A. pullulans*

A 5-day old slant was then used to inoculate the inoculum media which was incubated at 24 hrs at 30 ± 1 ^{0}C.The inoculumwas then transferred to major liquid broth (pH-5.5). Incubation was carried out at 30 ± 1 ^{0} C for 48 hrs.

A.pullulans growth on agar slant (3 days old)

Mycelial growth of *A.pullulans* in liquid broth medium

Enzyme Extraction:

After completion of 48 hrs of fermentation, the liquid broth containing mycelia growth of *A.pullulans* was subjected to centrifugation at 10,000 rpm for 15 min. at +4°C. The resultant pellet was repeatedly washed with chilled distilled water and finally using

citrate buffer (0.1 M, PH- 5.0). Residual mycelia mass obtained were then crushed in mortar-pestle using glass beads for around 30 mins under ice cold conditions. The resulaant homogenate was then centrifuged at 10,000 rpm for 15 min. at +4 ^{0}C. The supernatant thus obtained was used as the source of the enzyme.

Dialysis:

The crude enzyme was dialyzed against phosphate buffer (0.2 M, PH 7.0) for overnight duration. The dialyzed enzyme was used for the assay of fructosyltransferase.

Partial purification:

Partial purification of enzyme was done by ammonium sulfate fractionation. The enzyme was then further utilized to determine its kinetic behavior.

Enzyme Assay:

Fructosyltransferase assay was done using 60% sucrose solution as a substrate. The assay mixture containing 1ml of sucrose solution and 1ml citrate buffer was then incubated after the addition 1ml enzyme extract at 55 ^{0}C for 1 hr. A suitable control tube was also prepared along with the sample tube. The reaction progress was monitored spectrophotometrically at 540 nm using a suitable control. The enzyme activity was measured in terms of amount of glucose released in the reaction mixture using glucose oxidase peroxidase method.

Production and analysis of Fructooligosaccharides:

Production of FOS was carried out using a reaction mixture which consisted of 4 ml of 60 % sucrose in citrate buffer (pH 5.0) and 3 ml enzyme. The reaction mixture was incubated at 55°C for 18 h in a shaking water bath with regular sampling at 3 h intervals. At the end of incubation the reaction was arrested and the products in the reaction mixture were analyzed.

Chromatography:

In order to characterize the products formed by the action of fructosyltransferase on sucrose used as a substrate, paper chromatography was carried out. The composition of the solvent system used was: Ethyl acetate: isopropanol: water: pyridine (26: 14: 7: 2).

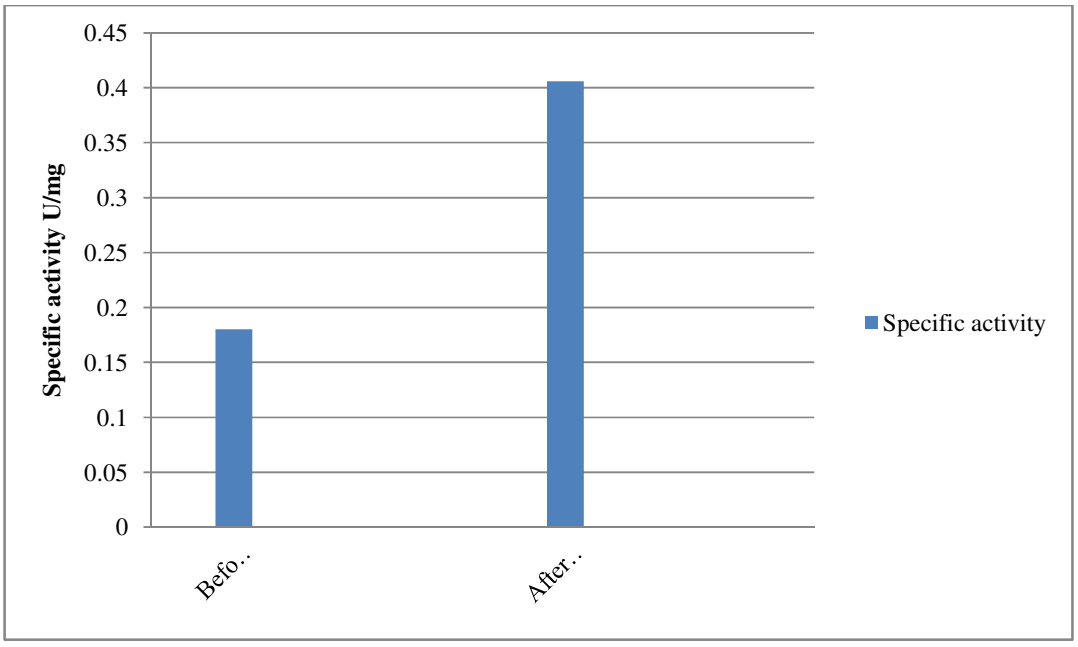
The reaction mixture along with standard sugar solutions [glucose, fructose, maltose, lactose, sucrose] were spotted on the Whatman No. 1 chromatography paper. The solvent system was allowed to run for 5 hours. Then chromatography paper was

removed from the chromatography chamber and it was air dried. The separated sugars were visualized by using Aniline diphenylamine as a spraying reagent.

Results & discussion:

Specific activity of enzyme before & after semipurification

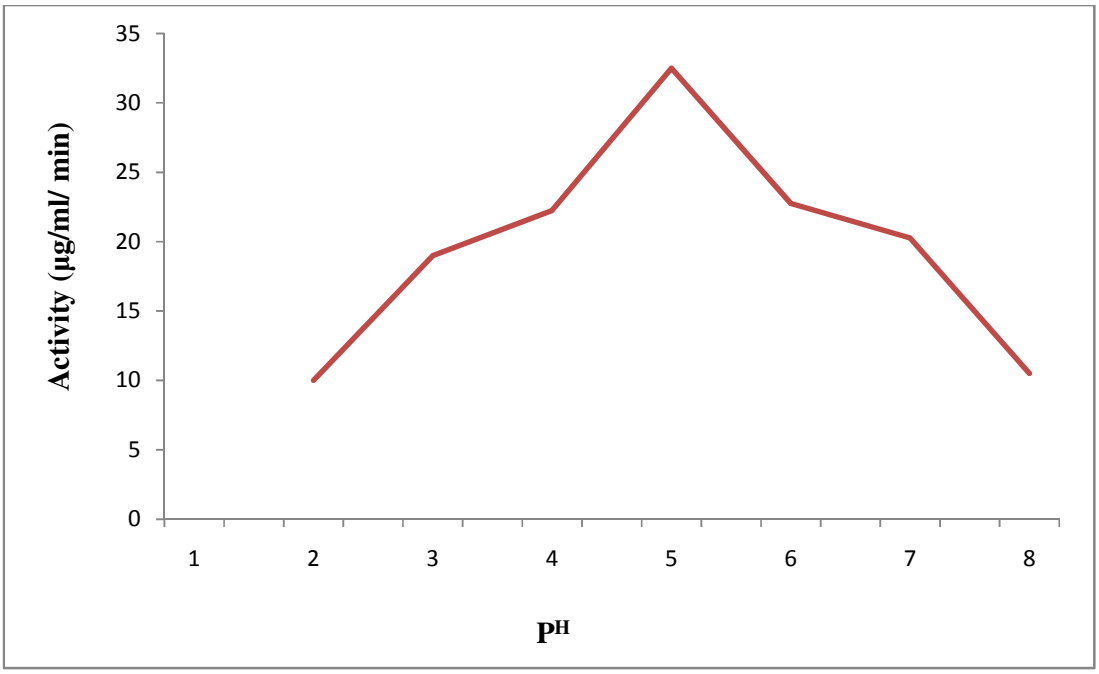
Fig.1 Specific activity of enzyme before & after semipurification



From the figue 1, it indicates that the specific activity of enzyme is enhanced after semipurification**.** Thus, the enzyme gets partially purified after ammonium sulfate fractionation as one of the preliminary purification technique.

Determination of Optimum pH of fructosyltransferase

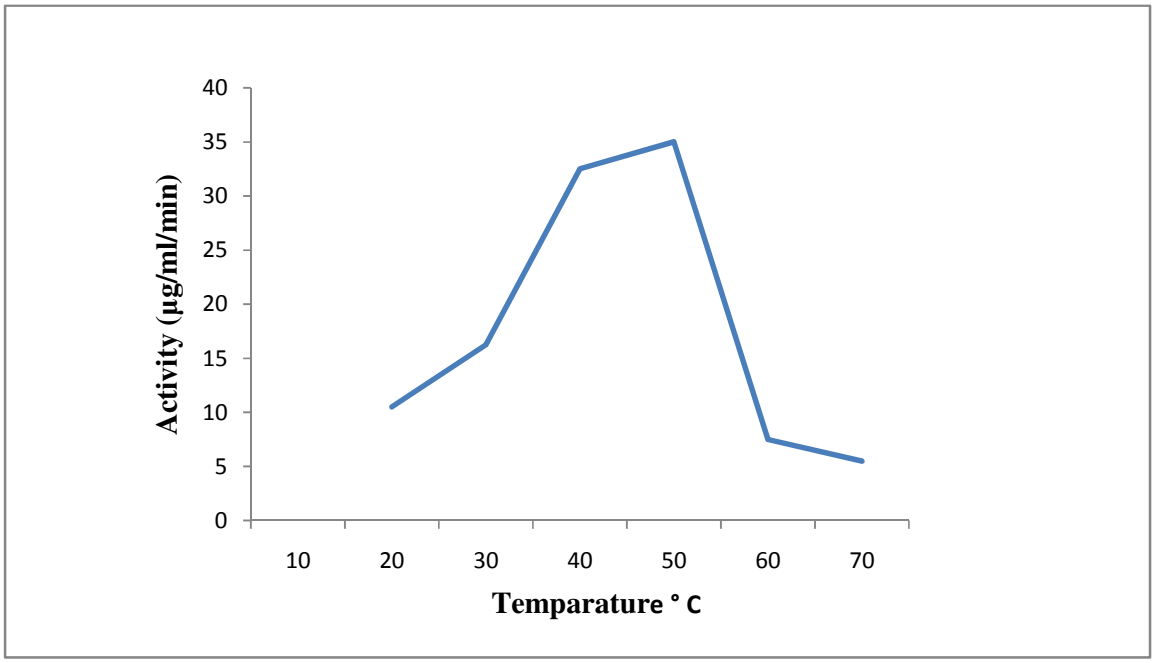
Fig.2. Determination of optimum pH of enzyme



The above graphical presentation concludes that the enzyme remains active between pH 2-7. After pH 7 the activity gets decreased exponentially. Maximal activity was observed between PH 4-6. Activity reached to the peak level at pH 5.0 indicating it as optimum PH of enzyme. The enzyme shows negligible activities towards extremes of acidic pH range i.e. 1.0 & alkaline pH range i.e. 8.0 and above.

Determination of Optimum temperature of fructosyltransferase

Fig.3: Determination of optimum temperature of enzyme



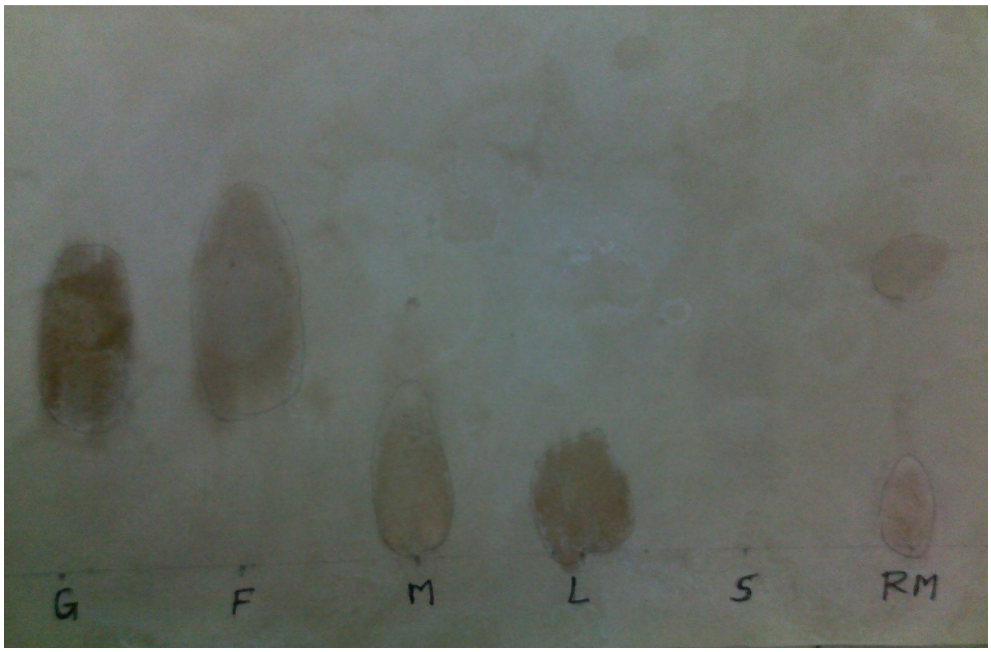
The above graph showed that the activity of fructosyltransferase increased along with increase in temperature from 20 °C to 50 °C. Exponential increase in the activity was noticed with increase in temperature from 30° C to 50 °C. The enzyme remained active between temperature ranges 20 °C to 60° C. Negligible activity was shown at 70° C and above. The optimum temperature for the enzyme observed was 50°C.

Table 1 Effect of Metal ions on the activity of fructosyltransferase

Metal salts	Effective conc. of metal ions (mM)	Enzyme activity µg/ml/min	% enzyme activity
Standard	10	35	100
$MnCl_2$	10	38	108.5
$FeSO_4$	10	31	88.5
$HgSO_4$	10	6	17.1
$MgSO_4$	10	37	106
NaCl	10	32	91.4
$CuSO_4$	10	30	86
$ZnSO_4$	10	31	88.5

Fructosyltransferase exhibited fluctuations in its activity level when the enzyme assay was carried out in the presence of various different metal ions. The observation table 1 displayed that $\mathbf{Mn^{+2}}$ & $\mathbf{Mg^{+2}}$ showing the maximal activity are **activators** of the enzyme. Whereas $\mathbf{Fe^{+2}}$, $\mathbf{Na^{+}}$, $\mathbf{Cu^{+2}}$ & $\mathbf{Zn^{+2}}$ reduces the activity of the enzyme. The enzyme activity was totally declined in the presence of $\mathbf{Hg^{+2}}$ indicating that $\mathbf{Hg^{+2}}$ is the strong **inhibitor** of the enzyme.

Paper Chromatography:



G=Glucose

F=Fructose

M=Maltose

S=Sucrose

RM=Reaction mixture (Enzyme: Fructosyl transferase from *A.pullulans*)

The reaction mixture obtained after the action of fructosyltransferase on sucrose was further analysed by paper chromatography. The results observed revealed the presence of glucose, fructose and some short chain fructooligosaccharides resulting from the action of the enzyme.

CONCLUSION:

The *Aureobasidium pullulans* fungus displays production of fructosyltransferase which is intracellular enzyme. The enzymes from both *Aureobasidium pullulans* exhibits maximum activity around acidic pH i.e.5.0 and at the temperature 50 ^{o} C. $\mathbf{Mn^{+}}$ and $\mathbf{Mg^{+}}$ metal ions are activators while $\mathbf{Fe^{+2}}$, $\mathbf{Na^{+}}$, $\mathbf{Cu^{+2}}$ & $\mathbf{Zn^{+2}}$ slightly reduce the activity of the enzyme while $\mathbf{Hg^{2+}}$ acts as a strong inhibitor of the enzyme. The reaction products formed by the action of the enzyme on sucrose were characterized from paper chromatography. The chromatographic results revealed the presence of glucose, fructose and the oligosaccharides which are the components of exopolysaccharides produced by *Aureobasidium pullulans* in the culture broth.

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