Advances In Multidisciplinary & Scientific Research
A Multidisciplinary & Interdisciplinary Journal
Volume 8, No 3, June 2022 Series

Production, Purification and Characterization of B-D-Fructofuranosidase from Fusarium oxysporum Isolated from Grape Juice and Soil Sample from Sugar Cane Dumpsite

[1]Onitilo V.O., [2]Sanusi J,F. & [1]Adebayo-Tayo, B.C.
[1] Dept of Microbiology, Faculty of Science, University of Ibadan, Ibadan, Oyo state, Nigeria
[2]Depat of Biological Sciences, Crescent University, Abeokuta, Ogun State, Nigeria
***Corresponding author:** +2348130051536; jadesolasanusi02@gmail.com

ABSTRACT

β-D-fructofuranosidase is an important enzyme needed in various industrial setup for the production of invert sugars. Production, purification and characterization of β-D-fructofuranosidase by *Fusarium oxysporum* under submerged fermentation were investigated. β-D-fructofuranosidase production by *Fusarium oxysporum* ranged from 36. 2- 65.8% and 7.2 -2 0.1% under different pH and incubation time. pH 7 and 7 days incubation time supported the highest production. β-D-fructofuranosidase production ranged from 16.5 - 44.0% and 27.8 - 68.8% when different organic and inorganic carbon sources were used. Potato peels and fructose supported the highest β-D-fructofuranosidase production. Peptone supported the highest β-D-fructofuranosidase production (37.9%) by *Fusarium oxysporum*. β-D-fructofuranosidase produced from *Fusarium oxysporum* was purified by Acetone precipitation method and gel filtration respectively. The crude and purified β-D-fructofuranosidase are 65.79% and 19.57%. pH 7, 35^{o}C and 30^{o}C incubation temperature, 2.0 mg/L of honey and molasses and 1.0 mg/L sucrose supported optimum activity of the crude and purified β-D-fructofuranosidase. The enzyme Kinetic parameters (K_m and V_{max}) are 0.1285mg/mL and 17.36U/mg. in conclusion, *Fusarium oxysporum* produced Invertase which can be harness for industrial uses.

Keyword: *Fusarium oxysporum*, Sucrose, Enzyme activity, Purification, Peptone

Aims Research Journal Reference Format:
Onitilo V.O., Sanusi J,F. & Adebayo-Tayo, B.C. (2022): Production, Purification and Characterization of B-D-Fructofuranosidase from Fusarium oxysporum Isolated from Grape Juice and Soil Sample from Sugar Cane Dumpsite. Advances in Multidisciplinary and Scientific Research. Vol. 8. No. 3, Pp 1–12. www.isteams.net/aimsjournal

1. INTRODUCTION

β-D-fructofuranosidase is an enzyme used for the hydrolysis of sucrose into inverted sugar which is a mixture of glucose and fructose (Uma *et al*., 2010; Ahmed, 2008). The product is named inverted sugar because of the change of rotation of sucrose to a negative value (-39.7^{o}) from a positive value (+66.5^{o}) when sucrose is hydrolyzed (Veana*et al*., 2018).It also catalyzes the breakdown of stachyose and raffinose (Shaheen*et al*., 2008). In addition, β-D-fructofuranosidasehas fructosyltransferase activity which is important for synthesis of short-chain fructo-oligosaccharide compounds.

This fact improves intestinal microflora and may prevent cardiovascular disease, colon cancer and osteoporosis (Linde *et al*., 2009). It is used in food industries for confectionery products, used in fermentation of molasses into ethanol, calf feed preparation, pharmaceuticals, manufacture of honey and plasticizing agents which are used in cosmetics (Essel and Osei; Ashokkumar *et al*., 2001; Kulshresthae*t al*., 2013). β-D-fructofuranosidase has also been reported to have profound human health benefits (Kulshresth a*et al*., 2013; Alvese*t al*., 2013; Elsayed and Enshasy, 2018). β-D-fructofuranosidase occurs widely in the biosphere and its presence has been reported in plants, certain animal tissues and micro-organisms (Kumar and Kesavapillai, 2012). Studies have shown that many organisms produce β-D-fructofuranosidase such as *Neurospora crassa, Candida utilis, Aspergillus niger, Aureobasidium* sp., *Lactobacillus reuteri, Fusarium oxysporum,* (Silveira *et al*., 2000; Uma *et al*., 2010; Kadowakie*t al*., 2013). β-D-fructofuranosidase can also be found in wild growing, on the skin of grapes and other fruits like Japanese Pear fruit (*Pyrusp.yrifolia*), Pea (*Pisum savitum*), Oat (*Avena sativa*), date fruit, carrot, potato, sugar cane and so on (Dahot and Noomrio, 1996; Uma *et al*., 2010; Romero-Gomez *et al*., 2002).

Traditionally, β-D-fructofuranosidase has been produced by submerged fermentation (Shaheen *et al*., 2008). β-D-fructofuranosidase can also be produced by solid state fermentation but only by the genus *Asp.ergillus only* (Balasubramaniem *et al*., 2001; Montiel-Gonzalez *et al*., 2004). Commercially, β-D-fructofuranosidase is bio-synthesized by yeast strains of *Saccharomyces cerevisiae* (Baker's yeast) or *Saccharomyces carlsbergensis* (Sivakumare*t al*., 2013).

In this study, production, purification and characterization of β-D-fructofuranosidase from *Fusarium oxysporum* isolated from sugarcane dumpsite using sucrose as the substrate was carried out.

2. MATERIALS AND METHODS

Culture and Preparation of Samples

Previously screened β-D-fructofuranosidase *producing Fusarium oxysporum* isolated from soil samples obtained from sugarcane dump sites and fermented grape juice were used during the study. The fungus was maintained on Czapek-Dox medium slant. The potatoes peels were dried in the oven, ground and then used as a substrate. The honey, molasses and date palm fruits were used as a substrate for enzyme production. The date-palm fruits were dried in a microwave and ground and then kept for further uses.

Production and assay of β-fructofuranosidase from the isolate

β-D-fructofuranosidase production by the isolate was done using Czapek-Dox broth. Sterile Czapek-Dox broth was inoculated with the fungus, incubated at 27^{o}C for 5 days under agitation. The filtrate from the culture was labeled as the crude enzyme. The isolate was further screened on Benedit's reagent by mixing 2mL cell free broth with 2mL Benedict's reagent. The green color, orange color and red color development indicate positive result (Sadashivam and Manikam *et al*., 1996). The filtrate from the culture was centrifuged at 6000 rpm for 15 minutes to obtain the supernatant which was used as the crude enzyme for β-fructofuranosidase assay. β-fructofuranosidase activity was measured by method described by (Bacon 1995). 1.00mL of the enzyme solution was added to 1.0mL sucrose (1% w/v) solution prepared in acetate buffer.

The reaction mixture was incubated at 60°C for 20 minutes and 3, 5-DiNitroSalicylic Acid (DNSA) solution (1.0 mL) was added to terminate the reaction. The absorbance was measured at 540nm using UV/VIS spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required for release 1µ mole of glucose/mL/minute under assay condition. Enzyme activity was expressed in International units. β-fructofuranosidase was calculated using this formula: IU/mL= concentration of glucose/0.5×30×0.180.

Purification of enzyme using Acetone precipitation and Gel filtration

The crude enzymes were purified using Acetone precipitation of proteins method. 30mL of the enzyme was dispensed into clean sterile conical flasks and treated with 120mL of cold acetone. The content were kept in the freezer for 1 hour for precipitation to occur an then centrifuge at 13000 rpm for 10 minutes. The supernatants were discarded carefully in order not to dislodge the precipitates. The tubes were uncapped for 30 minutes at room temperature in order to allow the evaporation of the acetone. The precipitates were re-suspended to an appropriate volume of cold Citrate buffer (pH 3.0) and kept in the freezer for 12 hours.

The precipitate obtained from acetone precipitation was introduced into Sephadex G-100 column (2.5× 90 cm) previously equilibrated with 0.1M Citrate buffer (pH 3.0) and kept overnight. The fractions were collected at a flow rate of 2mL per 10 min. The fractions containing enzyme (fraction 1-30) were pooled and stored at -10°C. The enzyme activity was determined using standard method.

Determination of total protein content of culture filtrates

The estimation of total protein content of culture filtrates was determined using the Biuret method (Gerardo *et al*., 2011). The biuret reagent was prepared appropriately, 200µL of sterile water as added to 750µL of the biuret reagent in a sterile bottles and 50µL of the fractions (enzymes) 1-30 were added into the mixtures. The reaction mixtures were incubated at room temperature in the dark for 30 minutes and the absorbance were taken at 540 nm using the UV/VIS spectrophotometer.

Effect of Production parameters on β-D-fructofuranosidase production Fusarium oxysporum

The effect of pH (2-9), incubation time (2-7 days), organic and inorganic carbon sources (potato peel, molasses, honey, coconut water, date-palm fruit, fructose, lactose, galactose, maltose and mannitol), and nitrogen sources (peptone, urea and ammonium chloride) on enzyme production was done by adjusting the pH, temperatures, substituting the sucrose and sodium nitrate in the basal medium with an appropriate carbon and nitrogen sources respectively. The sterile medium under a specified parameter was inoculated with agar plug of *Fusarium oxysporum,* incubated at 28°C for a specified day and then analyzed for enzyme activity.

Characterization of Crude and Purified β-D-fructofuranosidase produced by Fusarium oxysporum
Effect of different temperatures, pH, substrate concentration on enzyme activity

The effect of different incubation temperatures (20°C, 30°C, 35°C, 40°C and 45°C), pH (3.6, 3.8, 4.0, 5.0 and 5.6), different substrate concentration (molasses, honey and sucrose) (0.5 -2.0 %) on enzyme activity was determined using a standard methods. The reaction mixtures were incubated under appropriate conditions. The reactions were terminated by the addition of 1mL of DNSA. The absorbance was determined at 540nm using a UV/VIS spectrophotometer.

3. RESULTS AND DISCUSSION

Fusarium oxysporum produced 25.5% Invertase in the Basal medium used during this study. The effect of production parameters on β-D-fructofuranosidase production by isolate was investigated

Effect of pH on β-D-fructofuranosidase production by Fusarium oxysporum

The effect of pH on β-D-fructofuranosidase production by *Fusarium oxysporum* cultivated in submerged fermentation using sucrose as the carbon source is shown in Table I. The β-D-fructofuranosidase production ranged from 36.2%-65.79%. pH 7 supported the highest production of β-D-fructofuranosidase while the lowest β-D-fructofuranosidase production was recorded at pH 2. Of all the nine pH levels tested, pH 7 was the best pH that supported the highest β-D-fructofuranosidase activity after 7 days of incubation. Increased pH led to a reduction in the enzyme activity.
Enzymes are not stable towards alkaline conditions according to Balasundaram and Pandit (2001). According to them, the alkaline conditions result to the disruption of the enzyme.

Table 1: Effect of pH on invertase Production by *Fusarium oxysporum*

pH	Invertase Production (%)
2	36.2
3	51.9
4	47.6
5	61.7
6	42.0
7	65.8
8	46.8
9	58.3
10	56.6

Effect of Incubation time on β-D-fructofuranosidase production by Fusarium oxysporum

The effect of Incubation time on β-D-fructofuranosidase production is shown in Table 2. The β-D-fructofuranosidase production ranged from 18.08% -20.14%. The best incubation time for β-D-fructofuranosidase production was at day 7 of submerged fermentation while the lowest incubation time was at day 5. The best incubation time for optimal production of β-D-fructofuranosidase was 7 days. This implies that the organism was at its lag phase in which there was an upregulation of protein assembly, nucleotide metabolism, lipopolysaccharide biosynthesis and respiration which collectively led to cell division (Hamill *et al*., 2020).

Table 2: Effect of Incubation time on Invertase production by *Fusarium oxysporum*

Incubation time (Days)	Invertase Production (%)
3	18.1
4	8.6
5	7.2
6	9.4
7	20.1

Effect of Different Organic carbon sources on β-D-fructofuranosidase production by Fusarium oxysporum

The effect of different organic and inorganic carbon sources on β-D-fructofuranosidase production is shown in Table 3. The β-D-fructofuranosidase production ranged from 16.50% - 43.93%.The best organic carbon source for β-D-fructofuranosidase production was Potato peel which produced an enzyme activity of 43.93% through submerged fermentation while the lowest production was from Sugarcane juice.

The β-D-fructofuranosidase production ranged from 27.77%-56.23% when different inorganic carbon source was used. Fructose supported the highest β-D-fructofuranosidase production by the isolate while the lowest enzyme was produced when mannitol was used as an inorganic source of carbon. Sucrose was not the best inducer for β-D-fructofuranosidase production from *Fusarium oxysporum*. This is not in agreement with the report of Chauhan *et al*., (2016) who indicated sucrose as the best Carbon source.

Table 3: Effect of Organic Carbon sources on invertase production by *Fusarium oxysporum*

Organic Carbon Sources	Invertase Production (%)
Molasses	42.1
Date Palm	40.7
Honey	40.7
Potato peel	44.0
Sugarcane juice	16.5
Inorganic Carbon sources	
Lactose	36.8
Mannitol	27.8
Sucrose	28.0
Galactose	43.5
Fructose	68.8
Maltose	56.2

Effect of Different Inorganic sources on β-D-fructofuranosidase production by Fusarium oxysporum

The effect of different inorganic nitrogen sources on β-D-fructofuranosidase production is shown in Table 4. The β-D-fructofuranosidase production ranged from 27.20% - 37.90%. Peptone was the best inorganic nitrogen source for β-D-fructofuranosidase production through submerged fermentation while the lowest β-D-fructofuranosidase production was recorded when Urea was used. From this study, it was evident that peptone (1%) was an effective inducer for β-D-fructofuranosidase production by *Fusarium oxysporum*. Ability of peptone to support the highest Invertase production is not in agreement with the work of Chauhan *et al*., (2016) and Uma *et al*., (2010) that indicated yeast extract as the best Inorganic nitrogen source even though the micro-organisms used in their respective studies were different.

Table 5: Effect os Inorganic Nitrogen sources on invertase production by *Fusariumoxysporum*

Inorganic Nitrogen sources	Invertase Production (%)
Sodium Nitrate	28.0
Peptone	37.9
Urea	27.2
Ammonium chloride	37.8

Purification of β-D-fructofuranosidase

One liter of β-D-fructofuranosidase was harvested after 7 days of fermentation. Day 7 was selected because it supported the highest β-D-fructofuranosidase production. The crude enzyme was partially purified by using acetone. The enzyme activity of β-D-fructofuranosidase harvested on day seven after acetone precipitation is 19.57%.

Figure 1 shows the elution profile of gel filtration at the β-D-fructofuranosidase harvested on day 7 equilibrated with 0.1M Citrate buffer, pH 3.0. A peak showing the β-D-fructofuranosidase activity was acquired, they were pooled and characterized. Figure 2 shows the protein concentration of the partially purified enzyme.

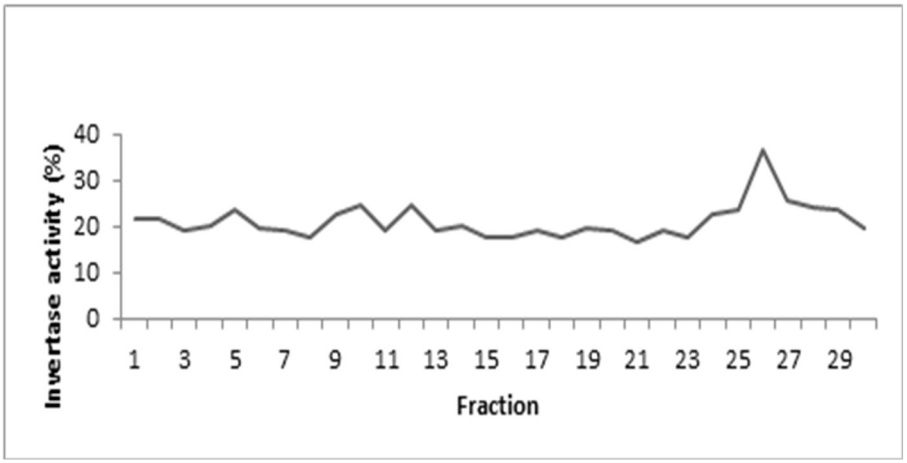

Figure 1a: Elution profile of β-D-fructofuranosidase (harvested on day seven of submerged fermentation) assayed in each of the fractions collected.

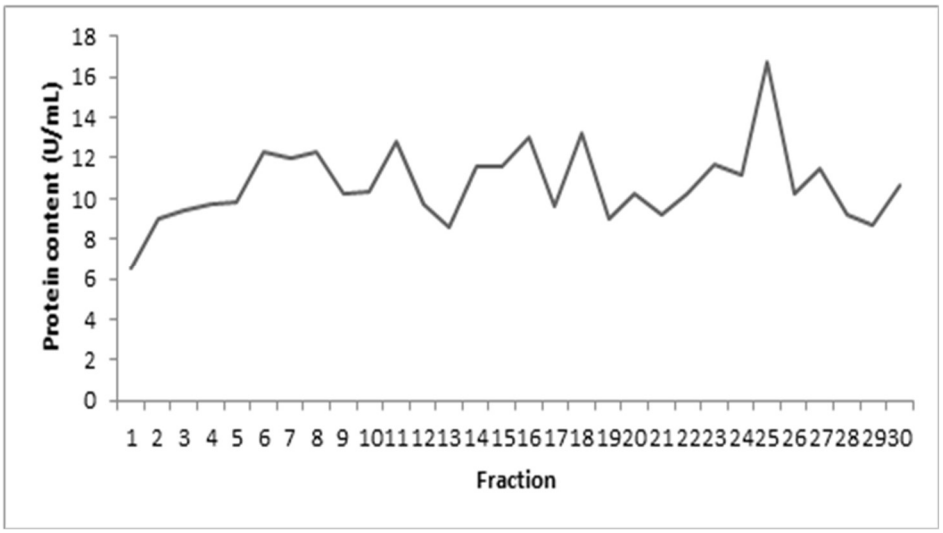

Figure 1b: Elution profile of Protein determination assay in each of the fractions collected.

Table 6: Partial purification of B-D-fuctofuranosidase by *Fusarium oxysporum* isolated from soil sample

Purification step	Volume mL	Units acitivity %	Total Activity	Protein mg/mL	Total protein mg/mL	Specific activity %	Yield (%)	Purification fold
Crude β-D-fructofuranosidase	100	65.79	6579	0.065	6.5	301.0	100	1
Purified β-D-fructofuranosidase	100	19.57	1957	0.115	11.5	224.6	29.74	1.76

Characterization of β-D-fructofuranosidase

The crude and the purified enzymes were characterized by checking the effect of pH, temperature and substrate concentration on enzyme activity. The effect of pH on β-D-fructofuranosidase activity is shown in Figure 2. pH 5.0 supported optimum activity of the crude enzyme while pH 4.0 was optimum for the purified enzyme. The best pH that supported optimum β-D-fructofuranosidase during the characterization of the crude enzyme was pH 5.0. This agrees with the work of Revathi and Uma (2014) and Oyedeji *et al*., (2017) even though the micro-organisms used for the research are different.

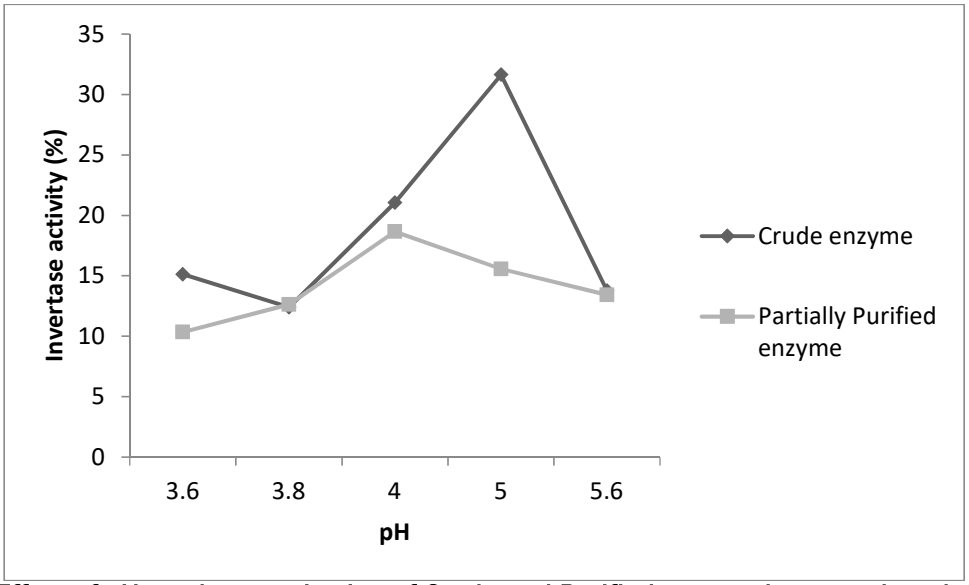

Figure 2: Effect of pH on characterization of Crude and Purified enzyme harvested on day seven of submerged fermentation.

Effect of Temperature on β-D-fructofuranosidase activity

The effect of temperature on β-D-fructofuranosidase activity is shown in Figure 3 for β-D-fructofuranosidase harvested on day 7. 35°C and 30°C supported optimum crude enzyme and purified enzyme activity (27.77% and 46.10%). An increase in temperature from 25°C to 35°C was accompanied by an increase in β-D-fructofuranosidase activity for the crude enzyme while the increase in temperature from 25°C to 30°C was accompanied by an increase in β-D-fructofuranosidase activity for the purified enzyme, beyond which the β-D-fructofuranosidase activity decreased. Optimum activity recorded at 35°C is in agreement with the work of Chelliappan and Madhanasundareswari (2014) and Oyedeji *et al*., (2017) who obtained the best enzyme activity at 35°C. Increase in temperature from 25°C-40°C led to a gradual increase in the enzyme production but a further increase in temperature led to an abrupt decline in enzyme production. This could be due to the fact that high temperatures cause denaturation of enzymes.

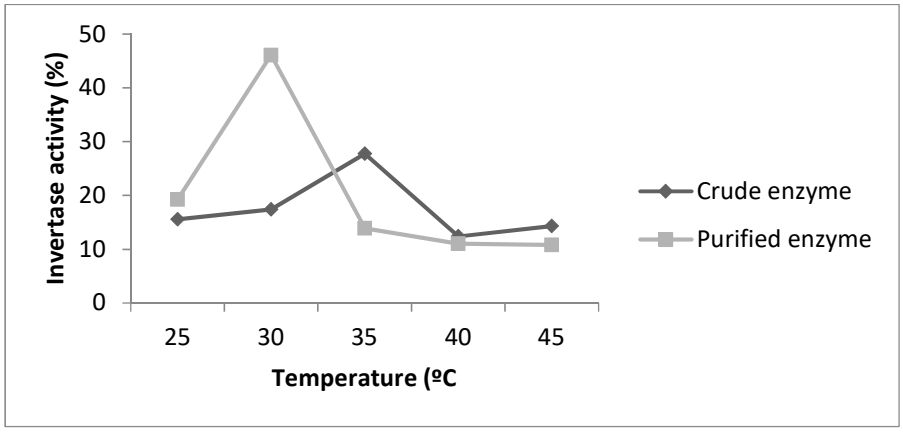

Figure 3: Effect of Temperature on characterization of Crude and Purified enzyme harvested on day seven of submerged fermentation.

The effect of different concentrations of honey, molasses and sucrose on β-D-fructofuranosidase activity is shown in Figure 4. β-D-fructofuranosidase activity in honey, molasses and sucrose ranged from 13.76 – 15.93%, 14.33 – 39.5% and 11.37 – 22.07 %. Optimum activity was recorded at 2.0 mg/L for honey and molasses and at 1.0 mg/L for sucrose. It was observed that the β-D-fructofuranosidase activity increased with increase in honey concentration. β-D-fructofuranosidase activity increased with an increase in molasses concentration. However, a further increase in concentration did not lead to an increase in β-D-fructofuranosidase production. It was observed that the β-D-fructofuranosidase increased with increase in sucrose concentration. However, a further increase in concentration did not lead to an increase in β-D-fructofuranosidase production.

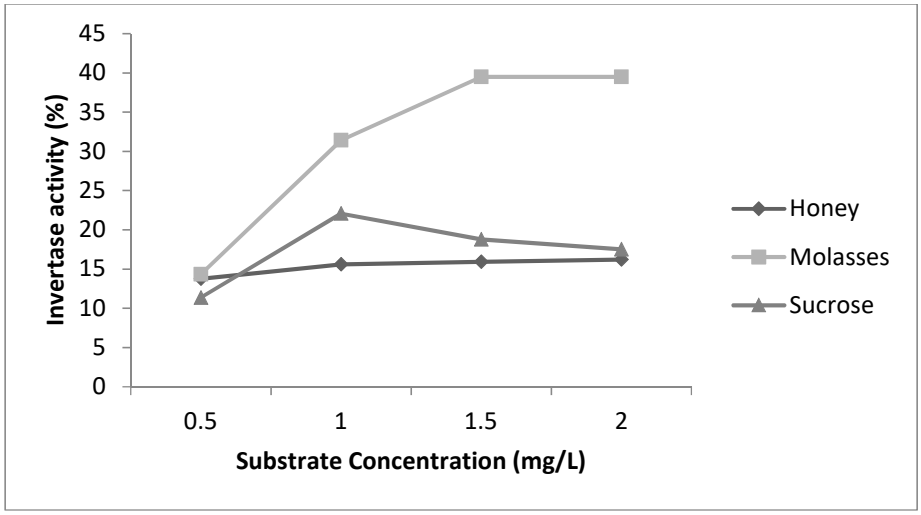

Figure 4: Effect of Different concentration of substrates on β-D-fructofuranosidase production.

In this study, the Kinetic parameters (K_m and V_{max}) were determined at 28°C for *Fusarium oxysporum* for concentrations ranging from 0.5-5.0% of honey as the best substrate. The K_m and V_{max} of *Fusarium oxysporum* are 0.1285 mg/mL and 17.36 U/mg. These values are not similar with those obtained from the work of Uma et al., (2010) who obtained 0.23 mg/mL and 15.8 U/mg respectively from *Aspergillus flavus* using sucrose as the substrate.

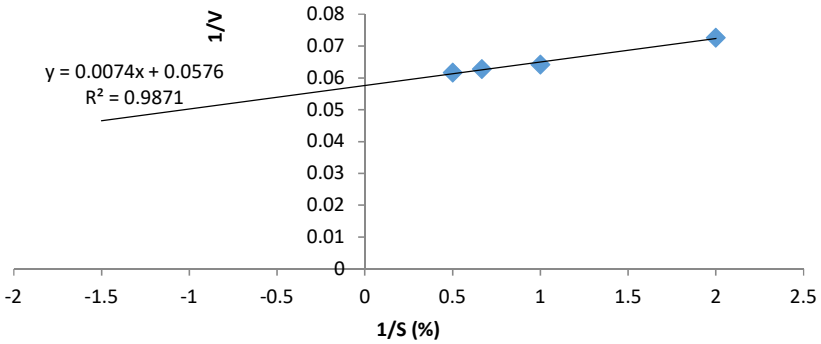

Figure 5: A Lineweaver-Burk plot for *Fusarium oxysporum*

In conclusion, Fusarium oxysporum produces Invertase, pH 7, 7 days incubation time, Potato peels, fructose and peptone supported the highest β-D-fructofuranosidase production. pH and 35°C and 30°C, 2.0 mg/L honey and molasses and 1.0 mg/L sucrose supported optimum activity of *Fusarium oxysporum* β-D-fructofuranosidase activity with a Kinetic parameters (K_m and V_{max}) of 0.1285mg/mL and 17.36U/mg.

REFERENCES

1. Ahmed, A.S. (2008). Invertase Production by *Bacillus macerans* immobilized on Calcium Alginate Beads. *Journal of Applied Sciences Research* 4:1777-1781
2. Alves, J.N., Joao, A., Luis, H.S.G. (2013). Production of invertases by anamorphic (*Asp.ergillus nidulans*) and teleomorphic (*Emericela nidulans*) fungi under submerged fermentation using rye flour as carbon source. Advances in Microbiology 3:421429
3. Balasundaram, B., Pandit, A.B. (2001). Significance of location of enzymes on their release during microbial cells disruption. *Biotechnology and Bioengineeering* 75 607-614
4. Balasubramaniem, A.K., Nagarajan, K.V., Paramasamy, G. (2001). Optimization of mediafor beta fructofuranosidase production by *Asp.ergillusniger* in submergedand solid state fermentation. *Process Biochemistry* 1241-124z
5. Bacon, J.S. (1995). Methods in Enzymology Volume I, and Academic press London, 258-262.
6. Benson, H.J. (1990). Microbiological Applications. A Laboratory Manual in General Microbiology 5th Edition *Brown Publishers* USA.Wm C 38-43
7. Chauhan, R.C., Chauhan, P.B., Gahlout, M. (2016). Isolation, Screening and Optimization of Invertase Production under Submerged Fermentation. KBS Commerce and Nataraj Professional College Volume III 2321-205
8. Chelliappan, B., Madhanasundareswari, K. (2014). Production and Optimization of Growth Conditions for Invertase Enzyme *by Asp.ergillus* sp. in Solid State Fermentation (SSF) Using Papaya Peel as Substrate. *Journal of Microbiology, Biotechnology and Food Sciences* 3(3): 266-269
9. Dahot, M.U., Noomrio, H.M. (1996). Purification and Some Properties of Invertases from Achrassapota fruit. *Journal of Islamic Academy of Sciences* 9:31-36
10. Elsayed, E.A., Enshasy, H.A. (2018). Effects of Different Aeration Rates and Feeding Strategies on Cell Growth and Invertase Production Kinetics by *Saccharomyces boulardii*. *Journal of Scientific and Industrial Research* 77:575-582
11. Essel, K.K., Osei, Y.D. (2014). Investigation of some kinetic properties of commercial Invertase from yeast. *Natural Products Chemistry and Research Open access Journals* 2:152
12. Hamill, P.G., Stevenson, A., McMullan, P.E., Williams, J.P., Lewis, A.D.R. Sudharsan, S.,Stevenson, K.E., Farnsworth, K.D., Khroustalyova, G., Takemoto, J.Y., Quinn, J.P., Rapoport, A., Hallsworth, J.E. (2020). Microbial lag phase can be indicativeof, or independent from,cellular stress. *Scientific Reports* Article 5948
13. Kumar, R. & Kesavapillai, B. (2012). Stimulation of extracellular invertase production from spent yeast when sugarcane press mud used as substrate through solid state fermentation. *Sp.ringer Plus*1:81
14. Linde, D., Macias, I., Fernandez-Arrojo, L., Plou, F.J., Jimenez, A., Fernandez-Lobato, M. (2009). Molecular and biochemical characterization of a β- fructofuranosidase from *Xanthophyllomyces dendrorhous*. *Applied and Environmental Microbiology*75:1065-1073

15. Montiel-Gonzalez, A.M., Viniegra-Gonzalez, G., Fernandez, F.J., Loera, O.(2004). Effect of water activity on invertase production in solid state fermentation by improved diploid strains of *Asp.ergillus niger*. *Process Biochemistry* 2085-2090
16. Oyedeji, O.M.K., Bakare, I.O.A., Olutiola, P.O., Omoboye, O.O. (2017). Optimized production and characterization of thermostable invertase from *Asp.ergillus niger* IBK1, using pineapple peel as alternate substrate. *Biocatalysis and Agricultural Biotechnology*9:218-223
17. Poonawalla, F.M., Patel, K.L. &Iyengar, M.R.S. (1965). Invertase Production by *Penicillium chrysogenum* and Other Fungi in Submerged Fermentation. *American Society for Microbiology* Volume 13, Number 5
18. Revathi, M. &Maheswari, N.U. (2014).Invertase Production from *Asp.ergillus* sp. under submerged fermentation. *International Journal of Universal Pharmacy and BioSciences*2319-8141
19. Romero-Gomez, S., Augur, C., Viniegra-Gonzalez, G. (2002).Invertase production by *Asp.ergillus niger* in submerged and solid- state fermentation. *Biotechnology Letters* 1255-1258
20. Sadashivam, Manikam. (1996). Evolution of invertase activity in honey over two years. *Journal of Agricultural and Food Chemistry*. 49:416-422
21. Shaheen, I., Bhatti, N.H. & Ashraf, T. (2008). Production, purification and thermal characterization of Invertase from a newly isolated *Fusarium* sp. under solid-state fermentation. *International Journal of Food Science and Technology* 1152-1158
22. Silveira, M.C., Oliveira, E.M., Carvajal, E. and Bon E.P. (2000). Nitrogen regulation of *Saccharomyces cerevisiae*invertase. Role of the URE2 gene .*Applied Biochemistry and Biotechnology* 84-86: 247-254
23. Talekar, S., Kate, A., Samant, N., Kumar, C. & Gadagkar, S. (2010). Australian Preparation and Characterization of Cross-linked Enzyme Aggregates of *Saccharomyces cerevisiae* Invertase. *Journal of Basic and Applied Sciences*4:4760-4765
24. Uma, C., Gomathi, D., Muthulakshmi, C. and Gopalakrishnan, V.K. (2010). Production, Purification and Characterization of Invertase by *Asp.ergillus flavus* Using Fruit Peel Waste as Substrate. *Advances in Biological Research* , Volume 4 Number 1, page 31-36
25. Veana, F., Adriana, C.F., Angela, M.G., Mariela, M.M., Lluvia, L.L., Pedro, A.Z., Juan, A.V., Raul, R.H. (2018).Invertase: An Enzyme with Importance in Confectionery Food Industry. *Enzymes in Food Technology* 187-212