

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

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## ABSTRACT

$\beta$ -D-fructofuranosidase is an important enzyme needed in various industrial setup for the production of invert sugars. Production, purification and characterization of  $\beta$ -D-fructofuranosidase by *Fusarium oxysporum* under submerged fermentation were investigated.  $\beta$ -D-fructofuranosidase production by *Fusarium oxysporum* ranged from 36.2- 65.8% and 7.2- 0.1% under different pH and incubation time. pH 7 and 7 days incubation time supported the highest production.  $\beta$ -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  $\beta$ -D-fructofuranosidase production. Peptone supported the highest  $\beta$ -D-fructofuranosidase production (37.9%) by *Fusarium oxysporum*.  $\beta$ -D-fructofuranosidase produced from *Fusarium oxysporum* was purified by Acetone precipitation method and gel filtration respectively. The crude and purified  $\beta$ -D-fructofuranosidase are 65.79% and 19.57%. pH 7, 35°C and 30°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  $\beta$ -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:**

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## 1. INTRODUCTION

$\beta$ -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°) from a positive value (+66.5°) when sucrose is hydrolyzed (Veanaet *al.*, 2018).It also catalyzes the breakdown of stachyose and raffinose (Shaheenet *al.*, 2008). In addition,  $\beta$ -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; Kulshrestha *et al.*, 2013).  $\beta$ -D-fructofuranosidase has also been reported to have profound human health benefits (Kulshrestha *et al.*, 2013; Alveset *et al.*, 2013; Elsayed and Enshasy, 2018).  $\beta$ -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  $\beta$ -D-fructofuranosidase such as *Neurospora crassa*, *Candida utilis*, *Aspergillus niger*, *Aureobasidium sp.*, *Lactobacillus reuteri*, *Fusarium oxysporum*, (Silveira *et al.*, 2000; Uma *et al.*, 2010; Kadowakiet *et al.*, 2013).  $\beta$ -D-fructofuranosidase can also be found in wild growing, on the skin of grapes and other fruits like Japanese Pear fruit (*Pyrus.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,  $\beta$ -D-fructofuranosidase has been produced by submerged fermentation (Shaheen *et al.*, 2008).  $\beta$ -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,  $\beta$ -D-fructofuranosidase is bio-synthesized by yeast strains of *Saccharomyces cerevisiae* (Baker's yeast) or *Saccharomyces carlsbergensis* (Sivakumaret *et al.*, 2013).

In this study, production, purification and characterization of  $\beta$ -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  $\beta$ -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 $\beta$ -fructofuranosidase from the isolate**

$\beta$ -D-fructofuranosidase production by the isolate was done using Czapek-Dox broth. Sterile Czapek-Dox broth was inoculated with the fungus, incubated at 27°C for 5 days under agitation. The filtrate from the culture was labeled as the crude enzyme. The isolate was further screened on Benedict'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  $\beta$ -fructofuranosidase assay.  $\beta$ -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 1 $\mu$  mole of glucose/mL/minute under assay condition. Enzyme activity was expressed in International units.  $\beta$ -fructofuranosidase was calculated using this formula: IU/mL= concentration of glucose/0.5 $\times$ 30 $\times$ 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 and 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 $\times$  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, 200 $\mu$ L of sterile water as added to 750 $\mu$ L of the biuret reagent in a sterile bottles and 50 $\mu$ L 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 $\beta$ -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 $\beta$ -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  $\beta$ -D-fructofuranosidase production by isolate was investigated

#### ***Effect of pH on $\beta$ -D-fructofuranosidase production by *Fusarium oxysporum****

The effect of pH on  $\beta$ -D-fructofuranosidase production by *Fusarium oxysporum* cultivated in submerged fermentation using sucrose as the carbon source is shown in Table 1. The  $\beta$ -D-fructofuranosidase production ranged from 36.2%-65.79%. pH 7 supported the highest production of  $\beta$ -D-fructofuranosidase while the lowest  $\beta$ -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  $\beta$ -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 $\beta$ -D-fructofuranosidase production by *Fusarium oxysporum****

The effect of Incubation time on  $\beta$ -D-fructofuranosidase production is shown in Table 2. The  $\beta$ -D-fructofuranosidase production ranged from 18.08% -20.14%. The best incubation time for  $\beta$ -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  $\beta$ -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  $\beta$ -D-fructofuranosidase production by *Fusarium oxysporum***

The effect of different organic and inorganic carbon sources on  $\beta$ -D-fructofuranosidase production is shown in Table 3. The  $\beta$ -D-fructofuranosidase production ranged from 16.50% - 43.93%. The best organic carbon source for  $\beta$ -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  $\beta$ -D-fructofuranosidase production ranged from 27.77%-56.23% when different inorganic carbon source was used. Fructose supported the highest  $\beta$ -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  $\beta$ -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                     |
| <b>Inorganic Carbon sources</b> |                          |
| Lactose                         | 36.8                     |
| Mannitol                        | 27.8                     |
| Sucrose                         | 28.0                     |
| Galactose                       | 43.5                     |
| Fructose                        | 68.8                     |
| Maltose                         | 56.2                     |

**Effect of Different Inorganic sources on  $\beta$ -D-fructofuranosidase production by *Fusarium oxysporum***

The effect of different inorganic nitrogen sources on  $\beta$ -D-fructofuranosidase production is shown in Table 4. The  $\beta$ -D-fructofuranosidase production ranged from 27.20% - 37.90%. Peptone was the best inorganic nitrogen source for  $\beta$ -D-fructofuranosidase production through submerged fermentation while the lowest  $\beta$ -D-fructofuranosidase production was recorded when Urea was used. From this study, it was evident that peptone (1%) was an effective inducer for  $\beta$ -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.

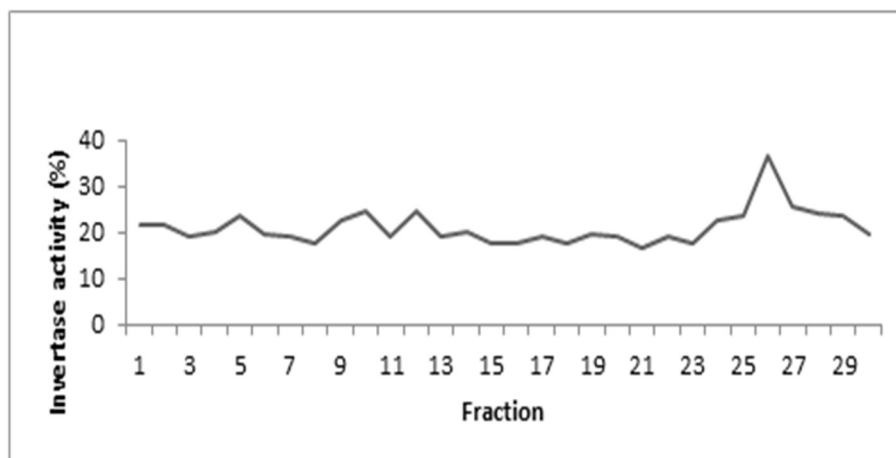
**Table 5: Effect os Inorganic Nitrogen sources on invertase production by *Fusariumoxysporum***

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

**Purification of  $\beta$ -D-fructofuranosidase**

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

Figure 1 shows the elution profile of gel filtration at the  $\beta$ -D-fructofuranosidase harvested on day 7 equilibrated with 0.1M Citrate buffer, pH 3.0. A peak showing the  $\beta$ -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  $\beta$ -D-fructofuranosidase (harvested on day seven of submerged fermentation) assayed in each of the fractions collected.**

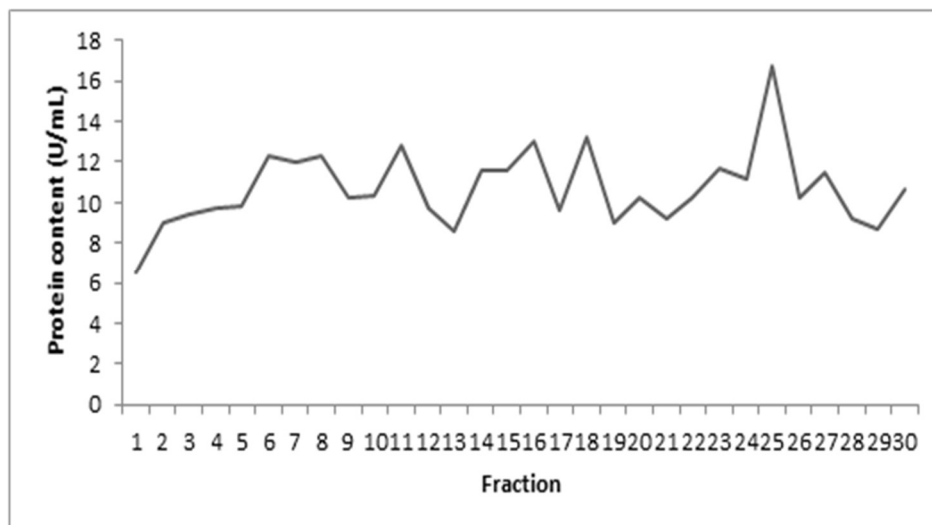


Figure 1b: Elution profile of Protein determination assay in each of the fractions collected.

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

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

#### Characterization of $\beta$ -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  $\beta$ -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  $\beta$ -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.

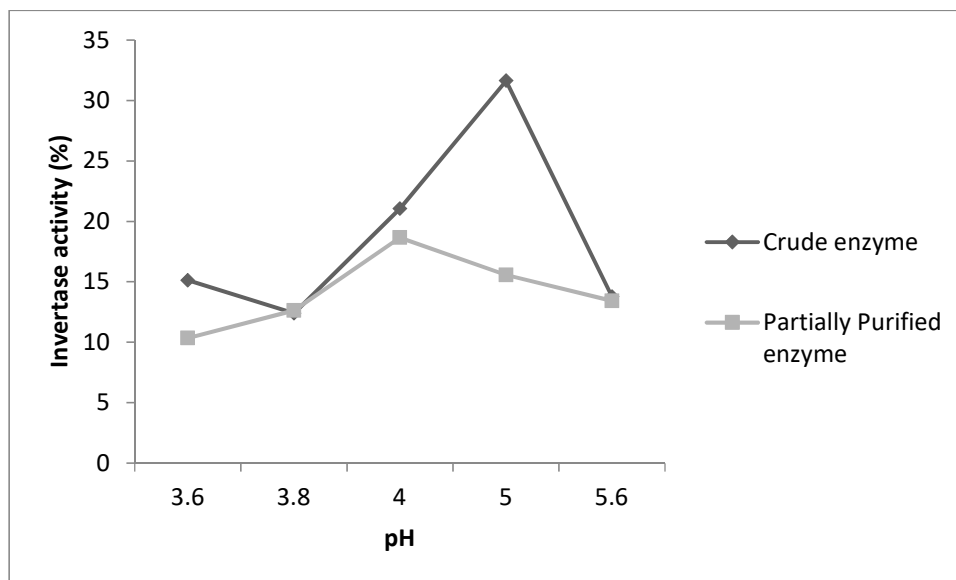


Figure 2: Effect of pH on characterization of Crude and Purified enzyme harvested on day seven of submerged fermentation.

#### Effect of Temperature on $\beta$ -D-fructofuranosidase activity

The effect of temperature on  $\beta$ -D-fructofuranosidase activity is shown in Figure 3 for  $\beta$ -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  $\beta$ -D-fructofuranosidase activity for the crude enzyme while the increase in temperature from 25°C to 30°C was accompanied by an increase in  $\beta$ -D-fructofuranosidase activity for the purified enzyme, beyond which the  $\beta$ -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.



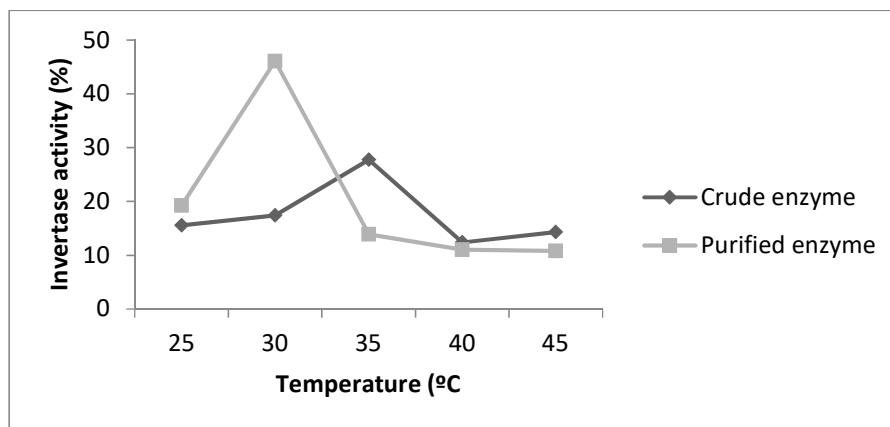


Figure 3: Effect of Temperature on characterization of Crude and Purified enzyme harvested on day seven of submerged fermentation.

The effect of different concentrations of honey, molasses and sucrose on  $\beta$ -D-fructofuranosidase activity is shown in Figure 4.  $\beta$ -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  $\beta$ -D-fructofuranosidase activity increased with increase in honey concentration.  $\beta$ -D-fructofuranosidase activity increased with an increase in molasses concentration. However, a further increase in concentration did not lead to an increase in  $\beta$ -D-fructofuranosidase production. It was observed that the  $\beta$ -D-fructofuranosidase increased with increase in sucrose concentration. However, a further increase in concentration did not lead to an increase in  $\beta$ -D-fructofuranosidase production.

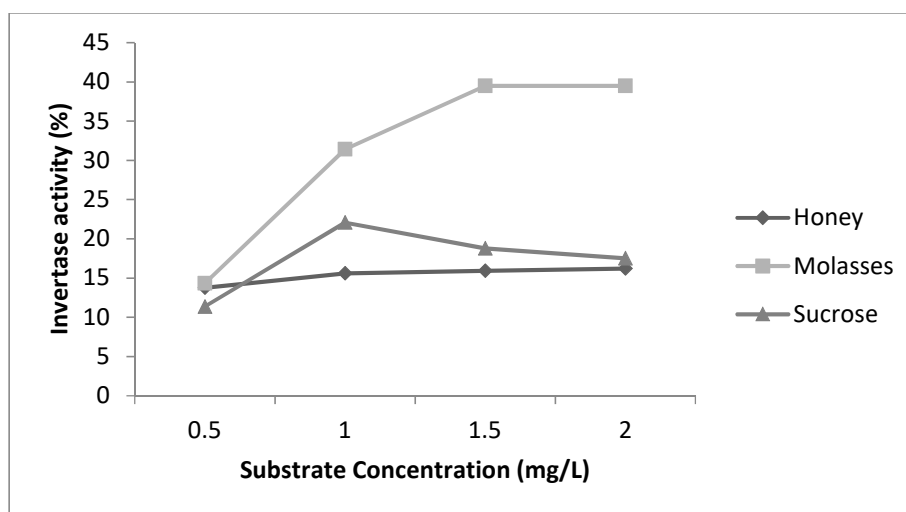


Figure 4: Effect of Different concentration of substrates on  $\beta$ -D-fructofuranosidase production.

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.

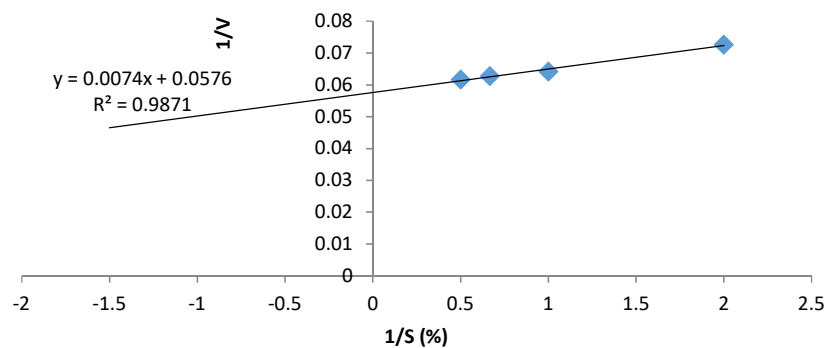


Figure 5: A Lineweaver-Burk plot for *Fusarium oxysporum*

In conclusion, *Fusarium oxysporum* produces Invertase, pH 7, 7 days incubation time, Potato peels, fructose and peptone supported the highest  $\beta$ -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*  $\beta$ -D-fructofuranosidase activity with a Kinetic parameters ( $K_m$  and  $V_{max}$ ) of 0.1285mg/mL and 17.36U/mg.

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