Plant Cell Biotechnology and Molecular Biology 22(37&38):252-265; 2021 ISSN: 0972-2025

THE OPTIMUM CONDITIONS FOR FRUCTOOLIGOSACCHARIDE PRODUCTION BY USING CRUDE FRUCTOSYLTRANSFERASE OF PLANT ORIGIN

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Article Information

Editor(s):
(1) Dr. Al-kazafy Hassan Sabry, Professor, National Research Centre, Egypt.
Reviewers:
(1) Tamaz Patarkalashvili, Technical University of Georgia, Georgia.
(2) Atif Ali, Hazara University, Pakistan.

Received: 05 April 2021
Accepted: 09 June 2021
Published: 21 June 2021

Original Research Article

ABSTRACT

Fructooligosaccharide (FOS) has an economic importance for its multiple uses in the food and pharmaceutical industries. FOS is produced naturally or industrially by enzymatic catalysis of a plant or microbial Fructosyltransferase (Ftase) enzyme. In the current study, the enzyme was extracted from plant sources, including Iraqi radish (*Raphanus sativus var. Longipinnatus*), Iraqi garlic (*Allium sativum*), Iraqi artichoke (*Helianthus tuberosus*), sweet potato (*Ipomoea batatas*) and Egyptian pineapple (*Ananas comosus*). The enzyme was extracted using potassium phosphate buffer at a pH range (6-8) in the presence of Cysteine, Triton x-100, and sodium acetate buffer at a pH range (4-7) in the presence of PMSF, EDTA and mercaptoethanol. Plant extracts showed the highest specific efficacy when using sodium acetate buffer at pH 6 for pineapple residues which was 200.732 units/mg protein. When studying the optimal conditions for FOS production using the crude enzymatic extract of the pineapple residues, which included (pH, temperature, concentration of the substrate, the concentration of the enzymatic extract and time), it was found that the optimal conditions for FOS production depending on the enzymatic activity were (6.5, 40°C, 40% and 250 µL and 18 hours respectively) as the enzymatic activity reached (271.32, 265.385, 266.615, 273.564 and 271.897) units/ml, respectively. By determining the enzymatic reaction products with Thin Layer Chromatography (TLC) and comparing the reaction products with standard sugars, it was found that the extract contains glucose, sucrose, 1-kestose, Nystose and FOS at Rf of (0.83, 0.65, 0.566, 0.48 and 0.41). Respectively, the presence of fructose was not observed.

Keywords: Fructooligosaccharide; fructosyltransferase; optimal condition; thin layer chromatography.

INTRODUCTION

Fructooligosaccharides (FOS) are short-chain polysaccharides of fructose sugar units with one molecule of glucose, consisting mainly of 1-Kestose, Nystose and 1-β- Fructofuranonystose, not digested in the small intestine but fermented by the action of endemic duodenal microbiology, especially Lactic acid bacteria, FOS is used in the nutritional and pharmaceutical fields because it has many characteristics, including low energy, non-carcinogenic, lowers phospholipid, triglyceride and cholesterol, as well as improving calcium and magnesium absorption, so it is beneficial for diabetics and can be used as a prebiotics [1-4].

FOS is produced naturally or artificially through enzymatic stimulation of Fructosyltransferase (Ftase) enzyme of plant microbial origin in the presence of sucrose as substrate, the enzyme transfers the Fructosyl group to the receptor which is sucrose or FOS [5].

Many studies indicated that plant Ftase consists of two different units (two enzymes) in molecular weight 60 - 85 KDa and 25-27 KDa, one of which decomposes the substrate which is sucrose into glucose and fructose, and the other enzyme works to transfer the fructosyl units to the receptor to form multiple sugar with chains ranging from 2 to 10 units accompanied by the release of glucose sugar, compared to microorganisms that have a single enzyme that forms FOS [1,4,6]. Ghazi et al. [7] indicated the presence of two enzymes used in the production of FOS, namely Ftase and β-Fructofuranosidase, or what is called the transforming enzyme "Invertase". Therefore, the study tended to determine the optimal conditions for the production of FOS by using the crude plant based Ftase enzyme.

MATERIALS AND METHODS

Plants

The study was conducted in the laboratories of the College of Agriculture / University of Basrah. The Iraqi radish (*Raphanus sativus var. Longipinnatus*), Iraqi garlic (*Allium sativum*), Iraqi mezze (*Helianthus tuberosus*), sweet potato (*Ipomoea batatas*) and Egyptian pineapple (*Ananas comosus*) were purchased from the local market of Basra Governorate - Iraq. Then it was transferred to the laboratory and cleaned of dust and washed well, the outer parts and peels were removed. Then, it was preserved in a temperature of 4°C until use.

Enzyme Extraction and Evaluation its Activity

The enzyme was extracted from the plants using several solutions as following:

Potassium phosphate buffer solution (0.05 M): Prepared at pH ranges (6, 6.5, 7, 7.5 and 8) in the presence of 5 g of Cysteine and 5 ml Triton x-100, with an amount of distilled water and adjust the pH to the optimal number mentioned above, then complete the volume to 1 litre with distilled water.

Sodium acetate buffer solution (0.05 M): Prepared at pH ranges (4, 4.5, 5, 5.5, 6 and 6.5) in the presence of 0.174 g PMSF and 0.07 ml mercaptoethanol with 0.292 g EDTA in an amount of distilled water and adjust the pH to the optimal number mentioned above, the volume completed to 1 litre with distilled water.

Sodium acetate buffer solution to measure enzyme activity: Prepared at a concentration of 0.1 M and pH 5.5, by dissolving 3.2812 g of sodium acetate with 3.4 ml of acetic acid in the presence of 196.822 g of sucrose with 0.2 g of sodium azide in an amount of distilled water, and adjust the pH to 5.5, the volume complete to 1 litre with distilled water.

Enzyme Extraction

The method of Wichienchot et al. [8] was used in Ftase enzyme extracting with some modifications. The enzyme was extracted from the plants using extraction solutions with different ranges of pH mentioned above. Each plant was taken separately and cut into small pieces, then mixed with pre-cooled extraction solutions in a 1: 1 mixing ratio, homogenised with an electric mixer for 2 minutes, the mixture was kept in a clean baker for 16 hours at a temperature of 4°C, then filtered with a piece of cloth, centrifuged at 10,000 cycles for 30 minutes at 4°C. The enzymatic activity and

protein concentration of each extract were estimated.

Enzyme Activity Estimation

The enzymatic activity of the extracts was estimated using the method described by Miller [9] and Ngampanya et al. [4], using dinitro salicylic acid (DNS) method and preparing a standard curve of glucose at a concentration (0-20) mg/ml. 100 µl of enzymatic extract was mixed with 400 µl of sodium acetate buffer solution at a concentration of 0.1 M in the presence of sucrose sugar at a concentration of 0.575 mol at pH 5.5 in test tubes and incubated at a temperature of 35°C for 24 hours. After that, 1 ml of the DNS reagent was added to the reaction mixture in dark test tubes, the tubes were placed in a water bath at 95°C for 5 minutes and then cooled to laboratory temperature, the absorbance was measured at a wavelength of 540 nm (Spectrophotometer-APEL. Japan). The unit of enzymatic activity was defined as the amount of enzyme that produces one micromole of glucose per minute under standard conditions.

Protein Concentration Estimation

The method of Bradford [10] was followed in estimating the protein concentration of enzymatic extracts.

Determination of the Optimum Conditions for FOS Production

Pineapple peels extract was chosen as the optimum source in producing FOS by having it the highest enzymatic activity of crude Ftase compared to other plant extracts, the method of Sangeetha et al. [11] and Mussatto et al. [12] was followed with some modification in determining the optimal conditions for FOS production, by measuring the enzymatic activity of the reaction time (0-24) hours, the pH (4-6.5), the temperature (30-80)°C and the concentration of the crude enzyme extract (10 -100) w/v and concentration of sucrose (20-80) w/v. The enzymatic activity was estimated when determining the optimal conditions according to the above-mentioned method.

Identification of Reaction Products using a Technique Thin Layer Chromatography

Aluminum plates coated with silica gel provided from Sigma, UK, according to the method described by Farag [13], with some modifications. A mixture of solvents consisting of acetic acid, chloroform and distilled water in a ratio of (0.5, 3, 3.5) (v/v) respectively was used, the spraying solution of sulfuric acid at a concentration of 40%, and the standard sugars (glucose, fructose, sucrose, 1-Kestose, Nystose and FOS) were prepared at a concentration of 0.1 g dissolved in 20 ml of distilled water, while 5 g of the sample under study was taken and dissolved in 19 ml distilled water. 1 µL of all standard samples and the crude extract was injected onto the TLC thin film after leaving the required distances from the sides and bottom. The plates were left in air for 30 minutes, then placed in the box containing the mixture of solvents. The samples moved to the top until they reached a height of 12.5 cm for a period of 90 minutes. Then the plates were taken and treated with a spray solution, then placed in the air for 30 minutes until dry. The RF distance for each sugar and for the samples according to the following equation: -

$$\mathbf{Rf} = \frac{Distance\ of\ the\ compound\ in\ the\ sample\ (cm)}{Distance\ of\ the\ compound\ (cm)}$$

Statistical Analysis

The results were analyzed using the SPSS [14] and followed the Complete Randomized Design (CRD), the results were compared using the lowest significant difference at a significance level of 0.01.

RESULTS AND DISCUSSION

Enzyme Activity Estimation of Plant Extracts

Fig. 1. shows the effect of extraction solutions at ranges of pH on the specific activity of Ftase, as sodium acetate buffer solution was used at pH (4, 4.5, 5, 5.5, 6 and 6.5) and potassium phosphate buffer solution at pH (6, 6.5 and 7, 7.5, and 8). The plant extracts showed a difference in the specific effectiveness values of both extraction solutions and the pH ranges used in the study, the Iraqi radish extract gave a range of specific

activity (11.888-32.155) units/mg protein and (10.555 -26.559) units/mg protein. For Iraqi garlic, it reached (25.073-49.334) and (16.434-53.994) units/mg of protein, while artichoke gave (67.8-133.156) and (30.212-135.786) units/mg of protein, sweet potatoes gave (78.699-138.63) and (30.321- 131.07) units/mg protein, while for pineapple peels (62.689-200.773) and (25.250-140.835) units/mg protein for the extraction solutions and the pH ranges used in the study, respectively. The reason for the discrepancy in the specific activity values among plant extracts may be attributed to several reasons, including the appropriate extraction solution and the appropriate pH and its ability to disengage the enzyme from plant tissues because the solution does not possess sufficient ionic strength.

This was confirmed by [8] who indicated that the enzyme extraction is affected by several factors, including the type of buffer extraction solution, the pH, as well as the degree of plant maturity. Segel [15] indicated that the difference in protein solubility in the extraction solution may positively or negatively affected on the activity of the enzyme. The results of the current study also showed a clear superiority in the specific effectiveness value of the Ftase enzyme extracted from pineapple peels using sodium acetate buffer solution at pH 6, as it reached 200.732 units/mg protein compared to the rest of the plant extracts. The reason for the increase can be explained by the suitability of sodium acetate at pH 6 to increase protein solubility, which positively affected on the activity of the enzyme, the addition of PMSF, EDTA, and mercaptoethanol also played a major role in increasing the specific activity through the ability of PMSF to inhibit protease enzymes, as well as the role of EDTA to bind some minerals that may hinder the enzyme's work. Through the foregoing, pineapple plant peels were selected as the best source for Ftase extraction to study the optimal conditions for FOS production.

Determination of the Optimal pH for FOS Production

The effect of different ranges of pH on the ability of pineapple peels crude extract in producing FOS depending on measuring of enzymatic activity was studied, as ranges of pH ranged from 4 to 7 were used for sodium acetate solution with a concentration of 0.1M containing 20% sucrose at an incubation temperature of 35 C. Fig. 2 shows a gradual increase in the efficacy values with an increase in the pH from 4 to 6.5 which was (79.592, 114.329, 135.835, 210.533, 263.922 and 271.320) units/ml respectively. With increase in pH, enzymatic activity increase from pH 4 to pH 6.5. As pH raise from 6.5 to 7 enzymatic activity sudden decrease to a limit of 138.859 unit/ml indicating that this type of enzyme prefers an acidic medium, while the decrease in the enzymatic activity with increasing the pH may be attributed to the fact that the medium is not suitable for the enzyme's work or it may cause changes in the active sites of enzyme action. Balasundaram and Pandit [16] and Nur Dini et al. [17] have argued that the reason for the decrease in the enzyme activity when the pH is increased may be due to the enzyme that is not stable in basic conditions leading to a decrease in the efficiency of converting the substrate (sucrose) to FOS. Koops and Jonker [6] reported that the best pH for FOS production using Ftase purified from the artichoke plant ranged from 5.5-7. Ngampanya et al. [4] and Ngampanya et al. [18] used a pH of 5.4 for the reaction medium consisting of potassium phosphate buffer solution with sucrose added in the presence of Ftase purified from the roots of the artichoke. Ende and Leare [19] found that the best efficacy of Fructosyltransferase was extracted from wild chicory. NurDini et al. [17] reported that the optimum pH for the action of the phytoenzyme used in the production of FOS and extracted from pineapple fruits was 5.5.

The Optimum Temperature for FOS Production

Temperature is one of the important factors that determine the production of FOS, as it increases collisions between the enzyme molecules and the base material, leading to an increase in the speed of the enzymatic reaction (15). During the reaction of the enzymatic extract with the substrate, ranges of temperature ranged from (30-60)°C were used in order to determine the optimum temperature for the production of FOS. The results shown in Fig. 3 showed an increase in the enzymatic activity values with increasing the temperature of the reaction medium, as it reached the highest enzymatic activity 265.385 units/ml at 40°C, to

begin with a gradual decline with the rise in temperature until it reached its maximum drop of 35.275 units/ml at 60°C. The reason for the increase in the enzymatic activity may be attributed to an increase in the temperature in the medium of the reaction to an increase in the kinetic energy of the enzyme and the substrate, and subsequently increasing the production until the optimum temperature is reached, then production decreases with increasing the temperatures due to the influence of the active site of the enzyme or to influence of the tertiary structure causing partial or entire damage for enzyme, leading to reducing or stopping the production. The results of the current study were similar to what was found by Wichienchot et al. [8], as it was found that the best temperature for the production of FOS from the enzyme Ftase purified from the amazot was at 35°C. While Koops and Jonker [6] note that the optimum temperature for FOS production medium using Ftase purified from amylation ranges from (20-35)°C. Whereas, NurDini et al. [17] indicated that the best temperature for FOS production from Phytoenzyme extracted from pineapple fruits was 60°C. Ngampanya et al. [4] also noted that the best temperature for FOS production using Ftase extracted from amazh tubers in the presence of sucrose as the reaction medium at pH 5.4 was 35ºC, indicating that the enzyme loses its activity at a temperature of 40-70 C, explaining the reason for this to the different locations Agriculture and the extent of plant adaptability to grow in areas of different environment. Judprasong et al. [20] and Tanjor et al. [21] have also suggested that the production of FOS using Ftase enzyme extracted from amazha tubers varies from one variety to another for a single plant. The results of the current study were similar to what was found by Wichienchot et al. [8], as it was found that the best temperature for the production of FOS from the enzyme Ftase purified from the artichoke was at 35°C. While Koops and Jonker [6] recognized that the optimum temperature for FOS production medium using Ftase purified from artichoke ranged from 20-35°C. Whereas, NurDini et al. [17] indicated that the best temperature for FOS production from phytoenzyme extracted from pineapple fruits was 60°C. Ngampanya et al. [4] also figured out that the best temperature for FOS production using Ftase extracted from artichoke tubers in the presence of sucrose as the reaction medium at pH 5.4 was 35ºC, indicating that the enzyme loses its activity at a temperature of 40-70°C, explaining the reason for that to planting sites variation and the extent of plant adaptability to grow in areas of different environment. Judprasong et al. [20] and Tanjor et al. [21] have also reported that the production of FOS using Ftase enzyme extracted from artichoke tubers varies from one variety to another for a single plant.

The Optimal Substrate Concentration in FOS Production

Fig. 4 shows the effect of using different concentrations of substrate which is sucrose (20, 30, 40, 50, 60, 70 and 80% w / v) on the efficacy of the crude enzyme extract Ftase in the production of FOS, as the highest enzyme activity reached 266.615 unit/ml at concentration 40% w/v of pure sucrose, with increasing the concentration, a decrease in the values of enzymatic activity was observed, as the maximum decrease at concentration 80% w/v with enzymatic activity of 72.410 units/ml. The reason for the decrease in activity with increasing the substrate concentration may be due to the saturation of the active sites of the enzyme and the inability to produce FOS, and it is believed that the increase in the concentration of the substrate causes an increase in the rate of the reaction due to the collision of the substrate with the enzyme, but the increase in the concentration above the normal limit in the medium of the reaction does not have any effects on the rate of the reaction and thus it does not become involved due to the complete saturation of the enzyme active sites, and this was confirmed by Fornandez et la. [22]. Also, NurDini et al. [17] stated that the access increase in sucrose concentrations beyond the required limit in the reaction medium does not cause any effects on the rate of production as the concentration becomes a non-specific factor for production and the enzymes become fully saturated with the substrate. The results of the study were similar to the findings of Wichienchot et al. [8], as it was found that the best concentration of sucrose used to produce FOS by Ftase enzyme extracted from the artichoke was 46%. This is less than what NurDini et al. [17] found, that the best

concentration of the substrate used to produce FOS by the enzyme purified from pineapple (pulp) is 60%, they also indicated that the producing of FOS does not depend mainly on the concentration of sucrose and the increase in sucrose concentrations above the required limit does not have any effects on the production rate as the concentration becomes a non-specific factor of production and the enzymes become completely saturated with the substrate [22].

Determination of the Optimal Enzymatic Extract Concentration for FOS Production

The results shown in Fig. 5 showed that there were significant differences between the values of the concentration of the enzyme extract used to produce FOS from Ftase enzyme from pineapple peels at a significant level of $P \leq 0.01$, as concentrations ranging (0-500) µL were used, as the enzymatic extract gave a gradual increase of enzyme activity with increasing the concentration to reach its maximum at concentration of 250 µL with enzymatic activity reaching 273.564 units/ml. With the increasing the enzyme concentration, the enzymatic activity decreased, as the maximum decrease was 92.974 units/ml at the concentration of 500 µL, and the reason for the decrease in the activity with increasing the concentration of the enzymatic extract may be due to the released glucose during the reaction that acts as a competitive inhibitor during production, and this what was confirmed by Antosova and Polakovic [5].

Jung et al. [23] and Yun et al. [24] were able to reduce the inhibitory effect of free glucose formed as a by-product in the stages of FOS production by adding Glucose Oxidase enzyme, which converts glucose into Gluconic Acid. NurDini et al. [17] concluded that the best concentration of the enzymatic extract of the enzyme Ftase obtained from pineapple is 30% in the production of polysaccharide, and no increase in the production of FOS was observed when concentrating of the enzyme, and the researcher also indicated that it is not possible to produce polysaccharide without the presence of the enzyme. Deepa et al. [25] found that when concentrating the enzyme above 6 units/ml, the maximum FOS production was reached in a relatively short time.

Optimum Time for FOS Production

Fig. 6 shows the determination of the optimal time for FOS production by adding the enzymatic extract to sodium acetate solution at a concentration of 0.1 M to which sucrose was added, as a slight increase was observed for (0-6) hours, amounting to (43.949, 56.462, 90.692 and 123.231) units/ml for the period [1,2,3,6] hours respectively, followed by a direct increase in the enzymatic activity for a period of [12,18] hours, reaching its maximum at 18 hours, after which a gradual decrease was observed for the period of 24 hours, reaching 256,633 units/ml, and the reason may be attributed to the slight increase of the period (0-6) hours resulted in the continuation of the enzyme binding with the substrate with the release of a small amount of FOS, while it increased steadily for 12 and 18 hours due to the occurrence of the reaction between the enzyme and the substrate in the production of FOS, which required a longer period of time, As time progressed, production decreased due to the saturation of the enzyme active sites with the substrate, and this what was confirmed by NurDini et al. [17]. Some studies have indicated that the optimal time for FOS production is (2-5) days, especially when using Ftase, which comes from microorganisms [11,26]. While Ngampanya et al. [4] noted that using 144 hours in FOS production by adding Ftase from artichoke root in 0.1 M potassium phosphate medium with addition of 0.46 M sucrose. While Wichienchot et al. [8] noted that the best reaction time for FOS production from Fructosyltransferase produced from the artichoke plant was 24 hours. NurDini et al. [17] found that the highest yields of FOS were obtained from the reaction of Phyto enzyme extracted from pineapple fruits in sodium acetate buffer medium (1M) for 100 minutes. The discrepancy between the results of our current study with other studies may be due to the possibility of a difference in the type of solution used or the type of plant and its degree of maturity. Some studies have also indicated that the activity of Ftase enzyme may be affected by the location of cultivation and the extent of plant adaptation, as well as the differences that may occur between one type and another of a single plant [4,20,21].

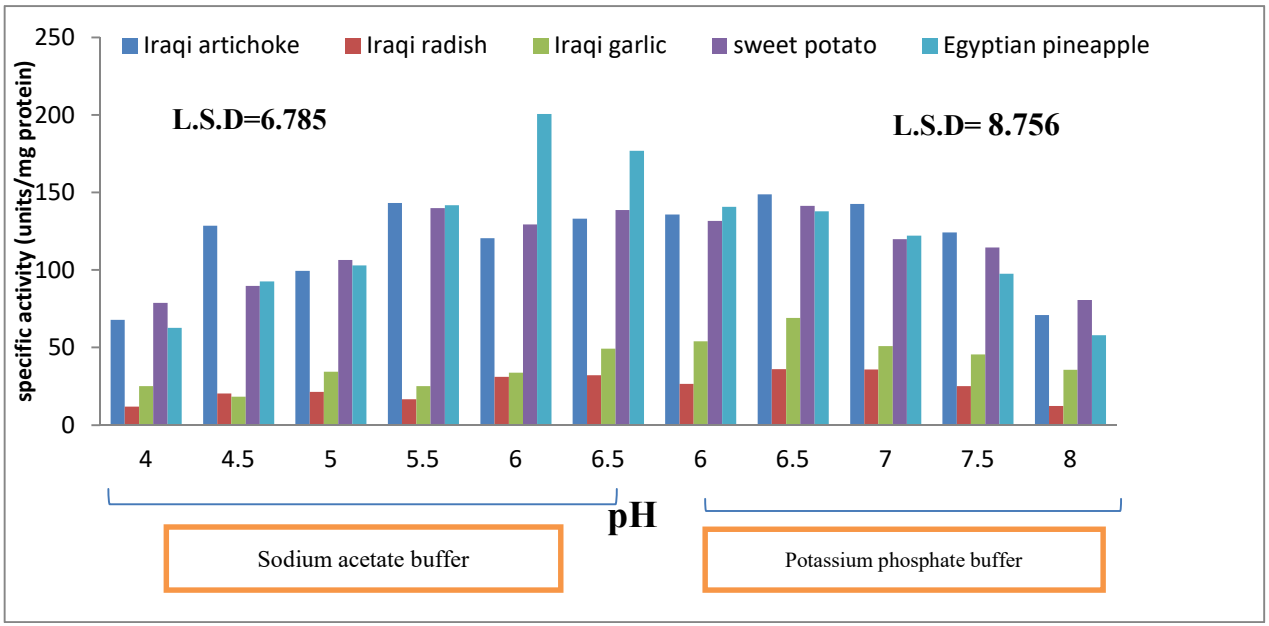

Fig. 1. Specific efficacy of plant extracts using different extraction solutions with different pH ranges

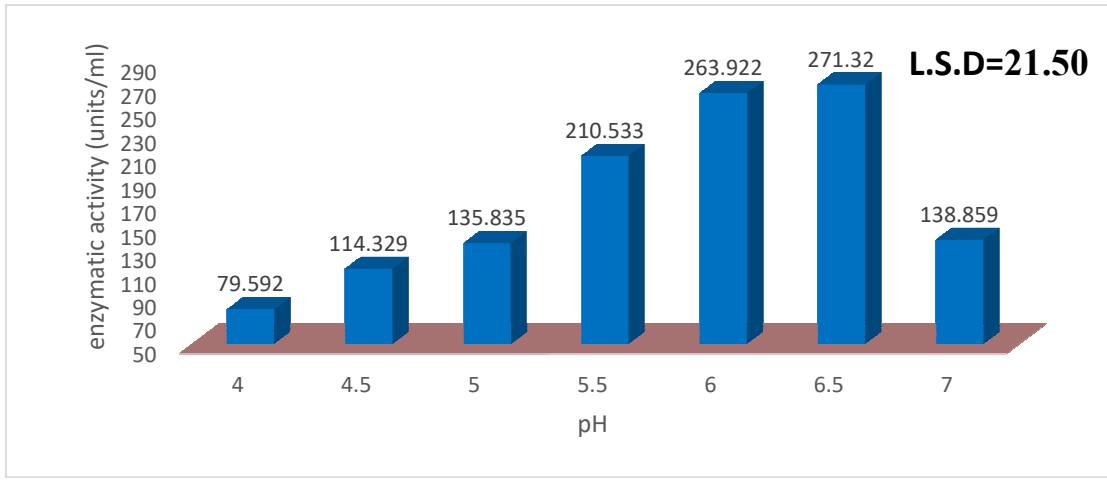

Fig. 2. Optimal pH for FOS production of ftase crude extract

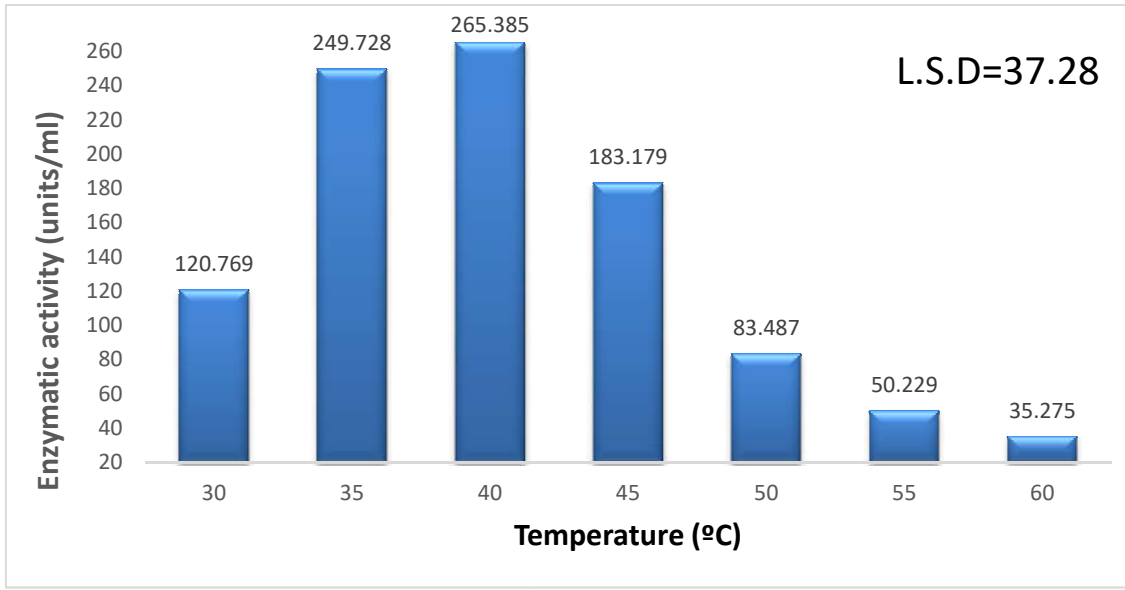

Fig. 3. The optimum temperature for FOS production using crude ftase from pineapple peels

TLC Identification Method

Thin-Layer Chromatograph (TLC) technique was used which is one of the important methods in chemical separation and detection of the components of the reaction products, which is inexpensive and simple and does not require long time to obtain results, as the separation process depends on the two fixed phases, which is the plate and the mobile phase (which is the liquid) as the sample decomposes according to its components and forming a colored spots, and by calculating the distance traveled by the spots with the calculation of time, it is possible to determine and know the composite materials of the sample [27], Results of Fig. 7 illustrate the use of TLC technique in determining the products of the enzymatic reaction (Ftase) of pineapple peels extract with the substrate (sucrose) in the medium of the reaction and comparing them with the

standard sugars, as it was found that the values of Relative Flow (**Rf**) of the standard sugars glucose, fructose, sucrose, nystose, 1-kestose, and fructosylnystose were (0.8, 0.784, 0.68, 0.48, 0.544 and 0.36), respectively.

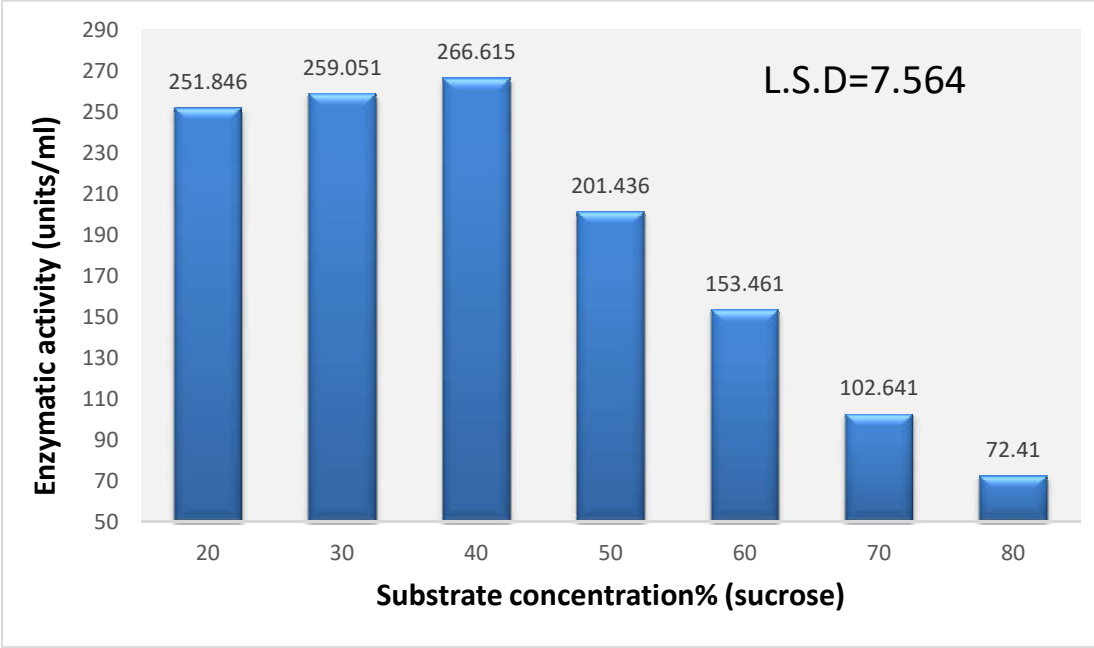

Fig. 4. Determination of the optimal concentration of substrate (sucrose) for FOS production

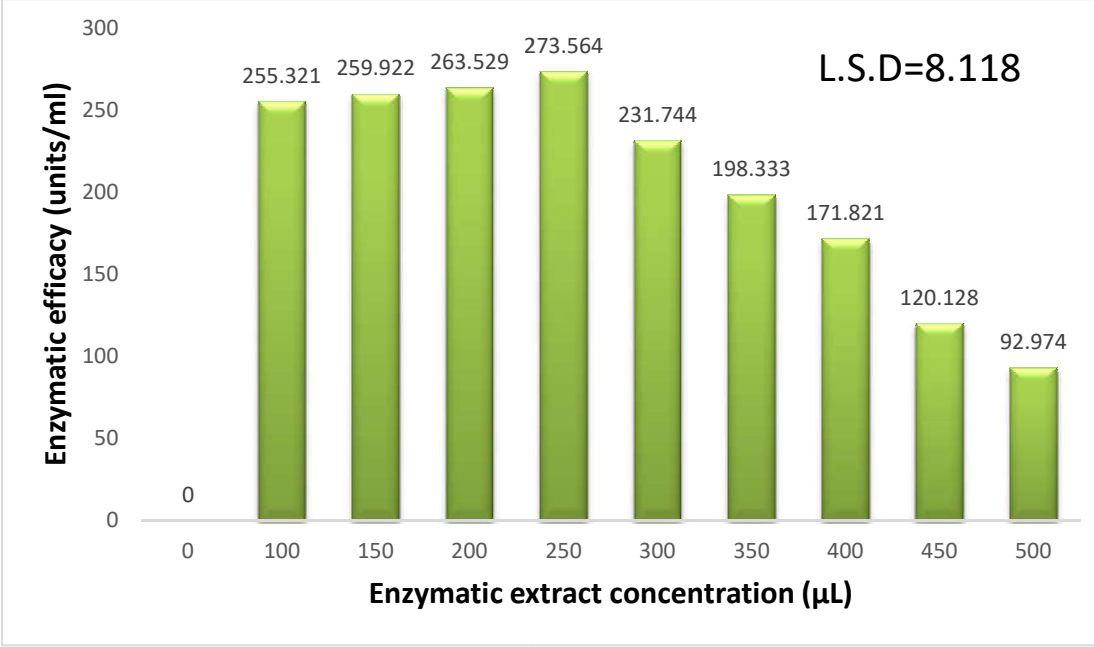

Fig. 5. Determination of the optimal concentration of ftase enzyme extract in FOS production

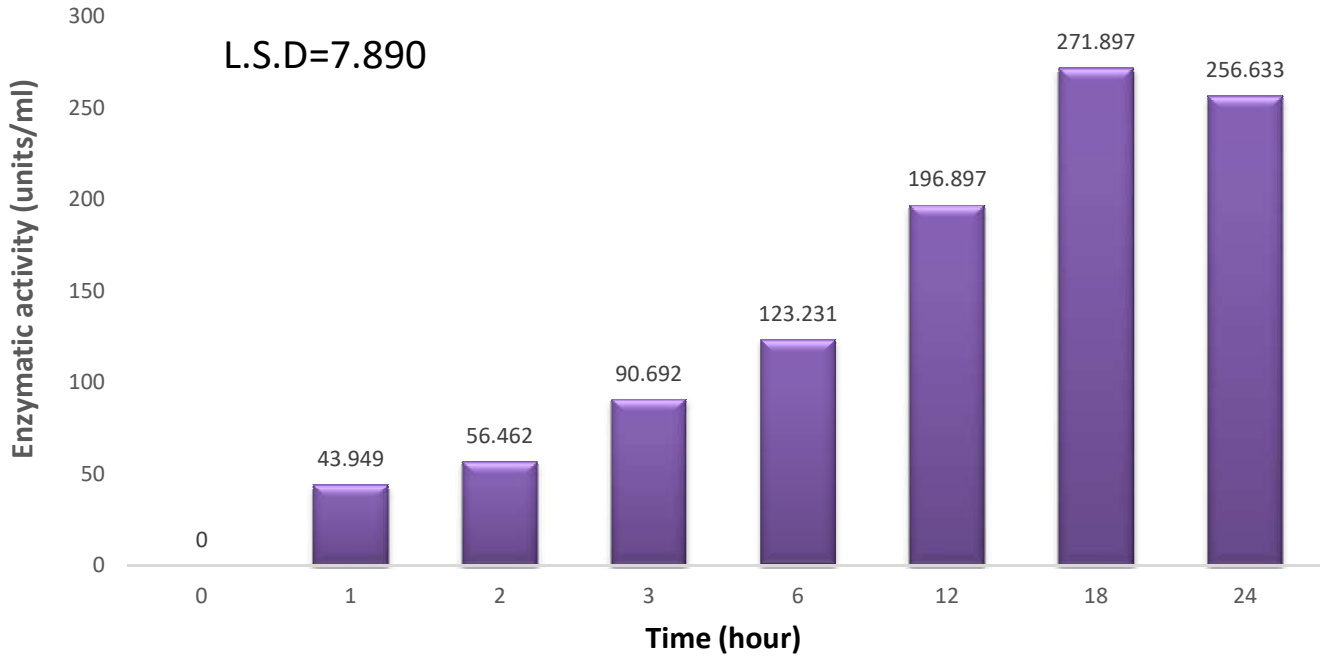

Fig. 6. Determination of the optimal time for FOS production using the enzymatic extract of ftase

While the sample under study showed six spots with an Rf value (0.83, 0.65, 0.566, 0.48 and 0.45) and when compared with the standard sugars, it showed a great convergence in the Rf value, as the sample's Rf results indicate the presence of glucose and sucrose, nystose, 1-kestose, and fructosylnystose respectively. Fructose was not observed, and the reason may be attributed to its connection to form reaction products. The results are consistent with Fontana et al. [28] who reported that the highest Rf value was glucose followed by fructose, sucrose and FOS, respectively, when detected by TLC. Lu et al. [29] indicated that glucose appears as the highest spot in the plate, followed by fructose, sucrose, and FOS, respectively when detected by TLC. Chikkerur et al. [30] was able to use TLC to detect sugars, by noting that glucose travels the highest Rf distance followed by fructose, sucrose, 1-ketose and nystose respectively. Antosova and Polakovic [5] indicate that commercial FOS contains 50-60% FOS, 25-30% glucose, 10-15% unconverted sucrose, and a low amount of fructose.

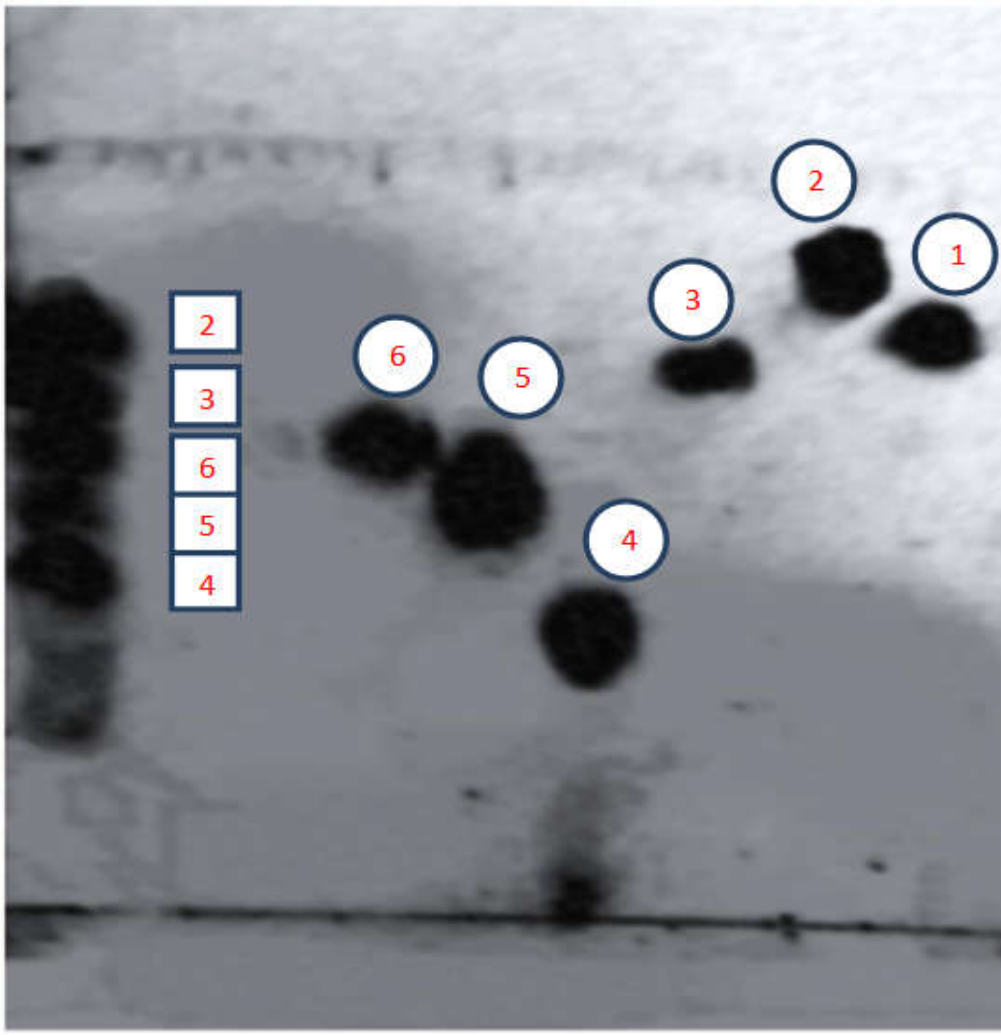

Fig. 7. Shows the visualization of sugars by TLC technique

The circle Shape represents Standers and the Square Shape represents Sample
1- Fructose 2- Glucose 3- Sucrose 4- FOS 5- Nystose 6- 1-kestose

CONCLUSION

Various plant sources were used to extract Ftase enzyme, including (artichoke, garlic, pineapple peels, sweet potato and radish) in the presence of sodium acetate and potassium phosphate extraction solutions at different pH ranges. Pineapple extract showed the highest specific activity in sodium acetate extraction solution compared to other plant sources used in the study. The optimum conditions for the production of FOS were determined by using raw pineapple extract as a source of Ftase enzyme, which included (pH, temperature, substrate concentration, the concentration of the enzyme and the optimal time for the production of FOS), as it was found that the best conditions for production were (6.5, 40°C, 40%, 250 µL and 18 hours). Also, when using the TLC technique to determine the products of the enzymatic reaction at optimum conditions for production, it was observed the exitance of Glucose, Sucrose, 1-Kestose, Nystose and FOS with different values of Rf, while the presence of fructose was not observed.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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