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Pectinase Effect on the Viscosity of Extracted Crude Thaumatin from the Aril of *Thaumatococcus daniellii* FRUIT.

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Abstract: *Thaumatin a natural sweetener extracted from the arils Thaumatococcus daniellii fruits. It is a very low calorie intensely sweet-tasty protein, a flavour modifier globally adjudged as sweetest substance on earth. The extraction of crude thaumatin is usually pose difficult due to the presence of the gel that normally encapsulates the protein within the fruit. To determine the optimal pH and temperature for extraction, crude thaumatin were extracted from 4,000mg of the sample at different pH values (2, 3, 4, and 5) in varied temperatures (25, 30, 40, 50, 55, 60, 70, 80 and 90^oC) each in 50ml volume of dilute sodium chloride (0.85M of aqueous sodium chloride). Further extraction were carried out from 4,000mg of the same sample in mixtures of different combined volumes of dilute sodium chloride and enzyme solution (0.1g/10ml pectinase) totaling 50ml at each extraction process. Ab initio mixture of 48ml of dilute Sodium Chloride and 2ml of enzyme solution were used for extraction of crude thaumatin. Several similar extractions were carried out at same determined pH value and temperature. Having subjected each extracted crude thaumatin to centrifugation at 10,000rpm for 1800secs, the decanted extracted crude thaumatin (supernatant) formed were, tested orally, assayed for protein via lowry method and viscosities measured with the aid of Oswald viscometer. The result findings revealed that the extracted crude thaumatin in mixture of 48ml of sodium chloride and 2ml of enzyme solution recorded minimal concentration of crude thaumatin of 23.0mg/ml but with maximal kinematic viscosity of 7,695.85mm^2/s, due to minimal hydrolysis of component of the gel made mainly of polysaccharide origin. Thus the most viscous extracted thaumatin of all the extracted crude thaumatin (supernatants) while extracted crude thaumatin in mixture of 38ml of sodium chloride and 12ml of enzyme extract recorded maximum concentration of crude thaumatin of 69.30mg/ml, with the least viscous medium as well as minimal kinematic viscosity of 4,803.45mm^2/s. This is due to optimal breakdown of pectin and other polysaccharides that formed the gel resulting to the release of maximal crude thaumatin. Therefore the extent by which the gel that encapsulate the sweetener can be hydrolysed and the water absorbing capacity of inhibited by combined effect of sodium chloride and enzyme solution. Consequently the extraction of the crude thaumatin eased substantially by introduction of combined volume of dilute sodium chloride and enzyme solution.*

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I. Introduction

Thaumatococcus daniellii benth is an economic plant with versatile uses in Southern Nigeria. The arils are attached to the seeds and contain the thaumatin, a non-sugar sweetener and taste modifier. Mature *Thaumatococcus daniellii* benth plant bears pale purple flowers and a soft fruit containing two or more shiny black seeds inside it. The fruit is fleshy, trigonal in shape and matures to a dark red/brown colour when fully ripe. At maturity each fruit contains three black, extremely hard seeds. Thaumatin, a very low calorie intensely sweet-tasty protein[1] (Raimi *et al.*, 2011), and the seeds are enveloped by a sticky thin, pale yellow basal aril, which contains the sweetening protein, thaumatin[2] (Lim, 2012). These seeds also produce a jelly that swells (entrap water) to 10 times its own weight and hence provide a substitute for agar[3] (Yeborah *et al.*, 2003), hence making it very difficult to extract the sweetener (thaumatin) from the fruits especially when extraction of thaumatin is carried out in distilled water.

The number of seed(s) usually one to three in a fruit determines the weight of fruits, normally 8 to 28g. The black seed has a hard seed coat secured by a slim layer of sticky, transparent gel. Attached to the black seed

is a smooth white soft, fleshy succulent cap called an aril arranged at the pinnacle of the seeds which contains the sweet substance called thaumatin. The aril is of an intensely sweet, nontoxic and heat stable protein, and thaumatin is extracted from it. Thaumatin is a recognized food additive and has potential in drug, confectionaries, and beverage manufacturing[4,5] (Sunderland *et al*., 2004; Swift *et al.*, 2002). Besides this natural sweetener however, a lot of artificial sweeteners has been implication in one source of adverse effect to the other in human body. Artificial sweeteners induce glucose intolerance by altering the gut micro biota[6] (Suez *et al.*, 2014).

II. Materials and Methods

The fleshy ripe *Thaumatococcus daniellii* benth fruits were harvested from *Thaumatococcus daniellii* plant from Otun village bush, Ayetoro local forest in Moba Local Government Area of Ekiti State, South-western Nigeria. The fruits were between the range of 1.4 – 2.1cm long, trigonal or pyramidal in shape, deep red and bright red colour as ripe fruits. The weight of fruits was within the range of 10 to 22g which depends on the number of seeds inside it. The *Thaumatococcus daniellii* benth fruits were identified by Forest Herbarium, Ibadan 110158. The fruits were transported to Covenant University, Otta Ogun State, where the fruits were thoroughly washed was with tap water followed by distilled water in order to remove any dirt or filth particles present on the surface, physically examined, then transported to biochemistry laboratory and stored at temperature of -18^0C.

Four hundred and twenty-two (422) of the fleshy fruits were selected and weighed; the total weight amounted to 6752g (6.752kg). Each of the fruits was peeled, frozen and freeze dried with freeze drier until the arils became brittle. By hitting the freeze dried fruits with hand repeatedly the brittle aril became separated from seeds and the rest of the fruits. Then the brittle aril was grind using an electronic blender into powdered form. A total weight of 400g (400,000mg) of powdered arils was obtained and stored in an air tight bottle as sample. Forty portions each of 4,000mg of the dried powdered sample were weighed, collected and stored for extraction of crude thaumatin.

2.1 Extraction and Determination of optimal pH and Temperature of Crude Thaumatin in sodium Chloride

After the preparation of exactly 0.85M of aqueous sodium chloride (sodium chloride), it was stored in two of 1000ml of conical flask. Four portions of 4,000mg of the sample were dissolved in 50ml of 0.85M of aqueous sodium chloride, after thorough shaking for 120secs at pH value of 2, 3, 4 and 5 each; 5mls each from the extracted thaumatin were pipetted into 10 empty test tubes. A total of forty (40) test tubes were collected and each was subjected to different temperature of 25^{o}C, 30^{o}C, 40^{o}C, 45^{o}C, 50^{o}C, 55^{o}C, 60^{o}C, 70^{o}C, 80^{o}C and 90^{o}C with the aid of a water-bath adjuster for 1200secs and thaumatin precipitated. Then at the end, 4ml of the raw crude thaumatin each was pipetted into 4ml micro tubes and centrifuged at 10,000 rpm for 1800secs. After the centrifugation the supernatants formed was decanted, tested orally and the remaining portions each was for assayed for protein.

2.1.1 Extraction of Crude Thaumatin in Mixture of different Volumes of Dilute Sodium Chloride and Enzyme Solution

Exactly 400ml of 0.1g/10ml of Pectinase (enzyme Solution) and 800ml of 0.85M of aqueous Sodium Chloride (dilute sodium chloride) were freshly prepared, and stored in 500ml and 1000ml of conical flask respectively. Eighteen (18) portions of 4,000mg of the samples each were weighed, collected and placed in 200ml conical flask each while crude thaumatin was extracted from each portion of the samples in mixture of different combined volume of dilute sodium chloride and enzyme solution totalling 50ml in each extraction process under same pH value and temperature of 3.0 and 55^{o}C respectively. Initially crude thaumatin was extracted from a portion of the samples in 50ml volume of 0.85M of aqueous Sodium Chloride with no volume of enzyme solution added. Then another crude thaumatin was extracted in a mixture of 48ml of dilute Sodium Chloride and 2ml of enzyme extract, followed by crude thaumatin extraction in a mixture of 46ml of dilute Sodium Chloride and 4ml of enzyme concentration. Several similar extractions were carried out under the same conditions in combined volume of the dilute sodium chloride and enzyme extract, totalling 50ml volume each as tabulated in table 1.0 below.

Then at the end of this extraction processes, 4ml each of the extracted crude thaumatin was pipetted into 4ml micro-tubes and centrifuged (GEN105 UV-VIs) at 10,000rpm for 1800secs, each supernatant was used for protein assay. Relatedly, the internal resistance of the fluid to flow under gravitational forces (viscosity) of each of supernatants were recorded with the aid of a viscometer on the supernatants resulted from the each extraction process.

Table 1.0 Extraction of Crude Thaumatin in Mixture of different Volumes of Dilute Sodium Chloride and Enzyme solution

Serial Number	Volumes of Dilute Sodium Chloride (ml)	Volumes of Enzyme Extract (Solution) (0.1g/10ml)
1	50	0
2	48	2
3	46	4
4	44	6
5	42	8
6	40	10
7	38	12
8	36	14
9	34	16
10	32	18
11	30	20
12	28	22
13	26	24
14	24	26
15	22	28
16	20	30
17	18	32
18	16	34

III. Results And Discussion

3.1 Crude Thaumatin Concentrations Extracted in Dilute Sodium Chloride at different pH Values in Varied Temperatures

The concentrations of crude thaumatin extracted in dilute sodium chloride at varied pH (range; 2.0-5.0) and in varied temperature (range; 25 ^{o}C to 90^{o}C) were determined by means of standard curve as shown in figure 1.0. The calculated values of protein in sodium chloride are shown in table 2.0, the values varied randomly, between 4.50mg/ml at 25^{o}C and 28.17mg/ml concentration of thaumatin at 55^{o}C.

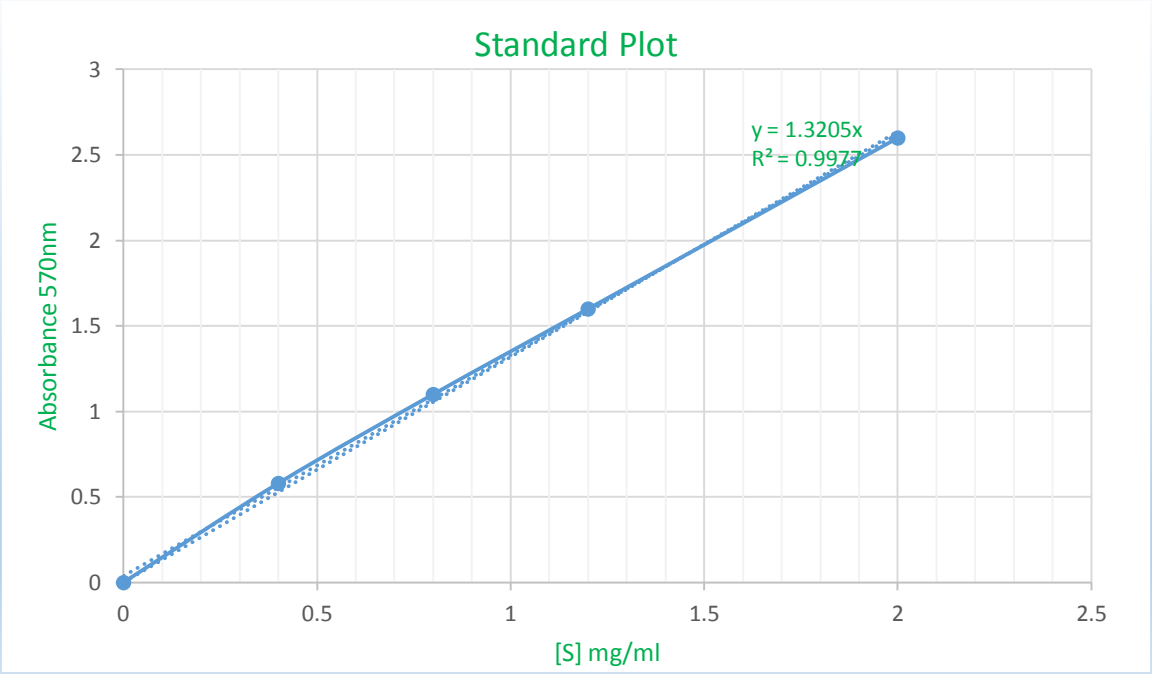

Figure: 1.0 Lowry Standard Plots for Protein Determination in Dilute Sodium Chloride

Table 2.0 Crude Thaumatin Concentrations Extracted in Dilute Sodium Chloride at different pH in varied Temperature

Temp. (0^oC)	pH 2.0		pH 3.0		pH 4.0		pH 5.0	
	Y (nm)	X (mg/ml)	Y (nm)	X (mg/ml)	Y (nm)	X (mg/ml)	Y (nm)	X (mg/ml)
25	8.8	6.70	9.20	7.00	6.90	5.20	5.9	4.50
30	11.2	8.47	13.2	10.00	8.60	6.50	8.0	6.10
40	20.6	15.60	21.8	16.50	18.2	13.8	16.4	12.4
45	23.0	17.40	27.6	21.00	19.8	15.0	17.4	13.2
50	26.2	19.80	29.1	22.00	23.8	18.0	22.4	17.0
55	33.2	25.14	37.2	28.17	28.2	21.35	26.6	20.14
60	21.4	16.20	23.4	17.70	19.8	15.0	20.0	15.0
70	19.1	14.50	21.8	16.50	17.0	12.8	16.8	13.0
80	16.4	12.40	21.5	16.30	15.6	11.8	14.4	11.0
90	16.2	12.30	21.0	16.00	15.0	11.3	14.2	11.0

Y (Absorbance) =1.3205X
X= Actual Conc.

Table 2.0 showed crude thaumatin extracted from the sample in dilute sodium chloride range from a minimal value of 4.50mg/ml at 25^oC to a maximum concentration value of 28.17mg/ml at 55^oC at pH values of 5 and 3 respectively. At pH of 2, minimum concentration of crude thaumatin extracted range from 6.70mg/ml at 25^oC to maximum concentration value of 25.14mg/ml at 55^oC. At pH 3, thaumatin extracted range from 7.00mg/ml at 25^oC to maximum concentration value of 28.17mg/ml at 55^oC. The concentration of thaumatin extracted at pH 4, range from 5.20mg/ml at 25^oC to maximum value of 21.35mg/ml at 55^oC, while at pH 5, thaumatin extracted range from 4.50mg/ml at 25^oC to a maximal value of 20.14mg/ml at 55^oC.

The table revealed that at 55°C, maximum concentrations of thaumatin was extracted at different pH values ranging from pH 2 to pH 5. From the table also maximum thaumatin was extracted at pH 3 while minimal concentration of thuaumatin was recorded at pH 5 at 25°C. This confirmed the optimal pH and temperature for extraction in dilute sodium chloride as pH 3 and 55°C respectively.

Crude Thaumatin Concentration Extracted in different volumes of Dilute Sodium Chloride and Enzyme solution

The concentrations of crude thaumatin extracted in different combined volume of 0.85M of aqueous sodium chloride (range: 50-14ml) and 0.1mg/10ml volume (range: 2-36ml) of enzyme solution at pH value of 3.0 at 55°C, were determined by means of standard curve (figure 2.0). Crude thaumatin concentration extracted varies randomly; the calculated values are shown in table 3.0. The least concentration (23.00mg/ml) of crude thaumatin extracted was recorded at combined volume of 48ml and 2ml of aqueous sodium chloride and enzyme solution respectively while maximum value (69.30mg/ml) at combined volume of 32ml and 18ml volume of aqueous sodium chloride and enzyme solution respectively.

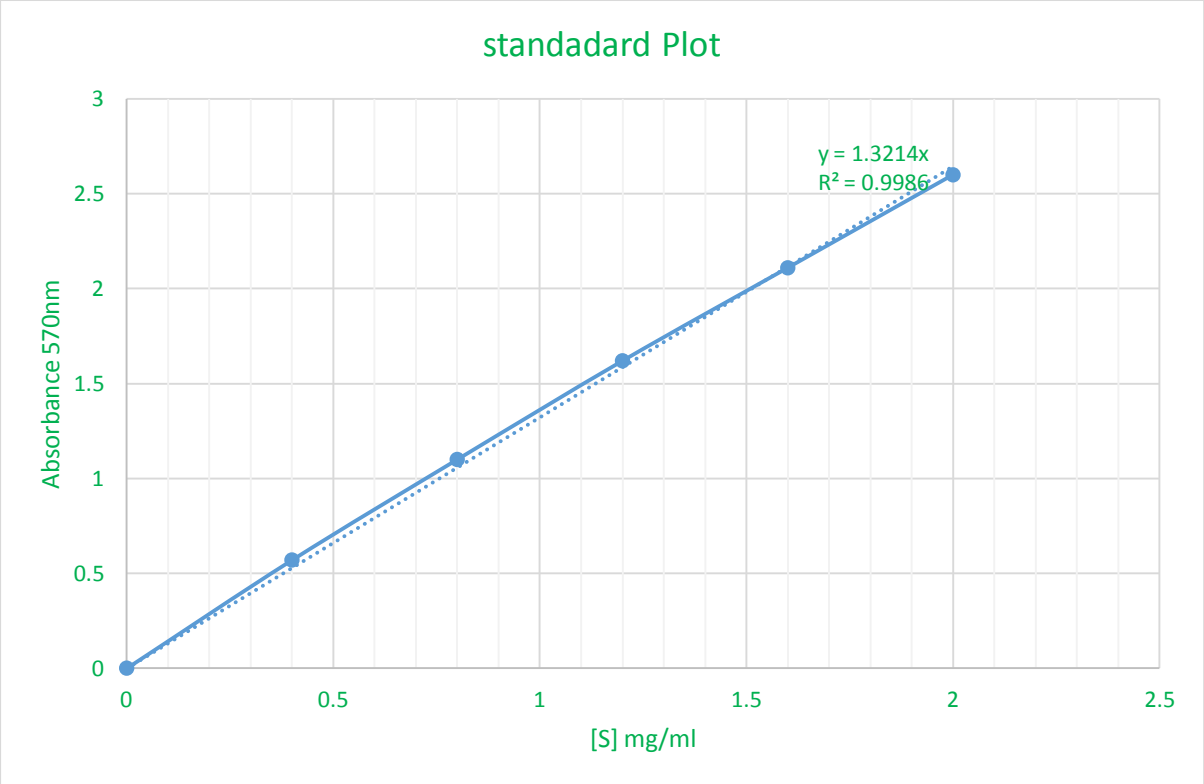

Figure: 2.0 Lowry Standard Plots for Protein Determination in different Combined Volumes of dilute Sodium chloride and Enzyme Solution

Standard Plots above is a protein assay (Lowry Method) having Bovine Serum Albumin as standard to determine the concentrations of protein at different absorbance of 570nm.

Table 3.0 Crude Thaumatin Concentrations Extracted in different Combined Volumes of Dilute Sodium chloride and Enzyme Solution

Enzyme Extract (0.1g/10ml)	$NaCl_{(aq)}$ (ml)	Y (nm)	X (mg/ml)
0	50	10.0	7.56
2	48	30.4	23.00
4	46	40.0	38.00
6	44	50.8	38.44
8	42	56.8	42.98

10	40	71.2	53.88
12	38	91.6	69.30
14	36	76.8	58.12
16	34	72.4	54.79
18	32	71.2	53.88
20	30	69.6	52.67
22	28	64.8	49.04
24	26	62.8	47.52
26	24	60.8	46.00
28	22	58.8	44.50
30	20	57.2	43.29
32	18	56.0	42.38
34	16	54.8	41.47
36	14	52.0	39.35

Y= 1.3214X

VISCOSITY COUNT RECORDED

The viscosity count value in each of the crude thaumatin concentrations extracted in combined volume of different enzyme solution and dilute sodium chloride were recorded as well as their kinematic viscosity calculated were presented in the table 4.0 as shown below. From the table the viscosity count recorded a maximum value of 293secs from crude thaumatin concentratin, extracted in uncombined volume of 50ml of dilute sodium chloride concentration while in a combined volume of 38ml of dilute sodium chloride and 12ml volume of enzyme solution recorded minimal value of viscosity count of 93secs.

Table: 4.0 Viscosities of Crude Thaumatin Extracted in different volumes of combined Dilute Sodium Chloride and Enzyme Extract (Solution)

Enzyme Extract (mg/ml)	$NaCl_{(aq)}$ (ml)	Viscosity Count Value (sec)	Kinematic Viscosity $(mm^2)/s$
0	50	293	15,133.45
2	48	149	7,695.85
4	46	138	7,127.70
6	44	134	6,921.10
8	42	114	5,888.10
10	40	111	5,733.15
12	38	93	4,803.45
14	36	98	5,061.70
16	34	101	5,216.65
18	32	104	5,371.60
20	30	105	5,423.25
22	28	108	5,578.20
24	26	111	5,733.15

28	24	114	5,888.10
30	22	117	6,043.05
32	20	118	6,094.70
34	18	119	6,146.35
36	16	121	6,249.65

Blank = 89
Constant = 51.65mm^2/s

Table 2.0 showed that minimum and maximum concentration of thaumatin extracted in dilute sodium chloride was at temperature of 25^{o}C and 55^{o}C respectively. At pH 2.0, thaumatin extracted from the sample in dilute sodium chloride was 6.70mg/ml and 28.17mg/ml, at pH 3, it was 7.00mg/ml and 28.17mg/ml, and 4.52mg/ml and 21.35mg/ml was recorded at pH of 4, while at pH 5, thaumatin extracted was 4.50mg/ml and 20.14mg/ml were recorded. From the table also maximum crude thaumatin extracted at pH 3 was higher at different temperatures compared to other pH values. Hence optimal pH and temperature for extraction of crude thaumatin in the dilute sodium chloride was pH 3 and 55^{o}C respectively. Therefore further extraction of crude thaumatin were carried out at pH 3 and 55^{o}C in varying volumes of sodium chloride and enzyme solution

However, it was observed that when the gel that encapsulate the aril that houses the sweetener is in contact with water, it swells many times thereby absorbing up to ten (10) times of its own weight of water[3] (Yeborah *et al*., 2003) and consequently causes problem in the extraction, since the gel absorbs both sweetener and distilled water (extractant). In dilute sodium chloride, much higher concentration of thaumatin crude were recorded, suggestive of the water absorbing capacity of the gel was remarkably inhibited when sodium chloride was used, making the gel to absorb lesser amount of the sweetener and the dilute sodium chloride (extractant), therefore ease crude thaumatin extraction substantially and markedly. It was also observed that at these pH values in varied temperatures, below 70^{o}C of extraction, the sweetness of the protein was fully retained when tested orally, also as opined by[7,8] Gibbs *et al*., (1996) and Lord, (2007). However, at 70^{o}C and above this temperature there was slight loss of sweetness which can be attributed to heat breakage or denaturation of disulphide bridges of the protein also stated by[9] (Higginbotham, 1979). Having recorded maximum concentration of thaumatin extracted in dilute sodium chloride at pH 3 and 55^{o}C. Hence same pH and temperature were also adopted in the extraction of crude thaumatin in different combined volumes of sodium chloride and enzyme solution.

Table 3.0 showed that the concentrations of crude thaumatin extracted **in different volumes of combined Dilute Sodium Chloride and Enzyme Solution** increased tremendously compared with the crude thaumatin concentration extracted from sodium chloride alone. This maybe as a result of the hydrolysis of the pectin-rich polysaccharide that forms most of the gel that were broken down by the enzyme (pectinase). From the table maximum concentration of crude thaumatin was recorded at combined of 36ml of sodium chloride and 12ml of enzyme solution, suggestive of maximum breakdown of pectin and other polysaccharides as a result of saturated kinetics[10] (Srinivan, 2020), therefore the hydrolytic effect of the enzyme was clearly obvious in the reduction of the viscosity count of the crude thaumatin extracted. It was also shown that further increase in the volume of enzyme solution beyond 12ml volume of enzyme solution and corresponding decrease in dilute Sodium Chloride added resulted to lesser yield in the concentration of the crude thuamatin recorded.

Having compared the different viscosities of crude thaumatin extracted in different combined volumes of dilute sodium chloride and enzyme solution as tabulated in table 4.0. The viscosity of crude thumatin when maximum concentration was recorded was extracted was the least counted value of viscosity as a result of saturated kinetics[10] (Srinivan, 2020), compared to others while the crude thaumatin with most viscous crude thaumatin recorded minimal concentration of crude thaumatin extracted.

This gradual decrease in the viscosity following the gradual decrease in the volume of dilute sodium chloride and the concomitant increase in the volume of enzyme extract added, resulted in the higher breakdown (hydrolysis) of long and complex molecules in the fruits pulp called pectin that occur as structural polysaccharides, (known to be responsible for the turbidity in the supernatants (extracted crude thaumatin) formed. Therefore total enzyme solution (extract) activity was determined by measuring the decrease in viscosity of pectin solution[11] (Gainvors *et al*., 2000). The gradual decrease in the viscosity counted was at the minimal level, in supernatant resulted from the extraction process of crude thaumatin in mixture of 38ml of dilute Sodium Chloride and 12ml of enzyme solution, an indication that the enzyme extract catalysing the hydrolysis of the gel (mainly pectin) at that concentration was at saturated kinetics[10] (Srinivasan, 2020). However, beyond this point of enzyme saturation, the supernatant form tend to be more viscous and slightly increases proportionately in viscosity as volume of the dilute sodium chloride added decreases while that of the enzyme solution increases as shown.

IV. Conclusion

From the present findings, extraction of crude thaumatin from *Thaumatococcus daniellii* benth fruit in sodium chloride yields relatively small amount of the sweetener, with relatively high viscosity. Consequently this renders the extraction of this *very low calorie intensely sweet-tasty protein* called thaumatin difficult due to the gel that encapsulate the sweetener. However by inhibiting the water absorbing capacity of the gel that encapsulate the sweetener with dilute aqueous sodium chloride in synergy with enzymatic hydrolysis of the pectin that constitute most of the polysaccharide forming the gel at pH of 3 and temperature of 55^{o}C, the jelly structure disintegrate consequently the viscosity of the extracted crude thaumatin drops following the breakdown of the gel resulting to higher concentration of crude thaumatin extracted.

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