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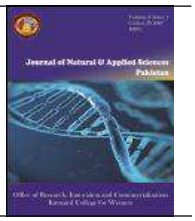

UTILIZATION OF INULIN-RICH PLANT EXTRACTS IN PROBIOTIC BACTERIAL CULTURE FOR LACTASE PRODUCTION

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Abstract

This research focuses on the isolation and identification of lactase producing probiotic bacteria in combination with inulin as a prebiotic, preparation of inulin rich garlic and onion extracts, and determination of the enzyme activity in the presence of these extracts. Streak plate method was used to obtain co-culture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* from yogurt in *Streptococcus thermophilus* isolation agar. After optimization of pH and temperature, lactase activity of the probiotic co-culture with 0.2%, 0.4% and 0.8% onion and garlic extracts in phenol red lactose broth was analyzed. A change in the pH of the culture from 6.6 to 3.5 was noticed at 42°C. Commercial inulin was used as positive control. Enzyme assay was performed for 48 hour culture by using Dinitrosalicyclic acid agent (DNS). Glucose standard curve was used as reference. Among three concentrations of inulin rich garlic and onion extracts used, high enzyme activity was obtained at 0.4% and 0.8%. The activity units obtained in the presence of garlic extract were 4.03 IU/ml/min and 4.29 IU/ml/min, with onion extract, the enzyme activity was 3.91 IU/ml/min and 4.03 IU/ml/min, respectively. Inulin-rich garlic extract seems to be a better choice for lactase enzyme production in the co-culture of the two probiotic strains.

1. Introduction

The persistent administration of probiotics is an approach that improves the health benefits of commensal microbes present in the gastrointestinal tract (GIT) of healthy humans. Probiotics in consolidation with prebiotics, also have health benefits by producing certain products that arise from anaerobic fermentation. In a healthy stomach *Lactobacillus*, *Streptococcus* and *Helic obacter pylori* are dominant. The frequency of microbes increases with passage down the GIT. The density found in small intestines is 10^3-10^6 cfu/mL which promotes the growth and survival of *Streptococcus and Lactobacillus* (Griffin P., 2011). Probiotic bacteria are found in the intestines of humans and other mammals where they improve host health by supplying nutrients and cofactors, positively encountering the pathogens, and stimulating host immune system as a response to the production of specific polysaccharides. Probiotic is defined as living, non-toxigenic organism that improves human host's health. Originally, probiotic was characterized as a product synthesized by one microbe promoting the development and multiplication of another microbe. Typically used probiotics are *L. reuteri, L. rhamnosus*, *Bifidobacterium* and some strains of *B. coagulans, L. acidophilus*-group, *L. casei*, *E. coli* DSM 6601 (German Collection for Microorganisms), the yeast *S. boulardii* and certain *Enterococci*, especially *E. faecium* SF68 (Anbukkarasi K. et al, 2014 and Barbara G. et al, 2016).

Prebiotics are poorly processed polysaccharides and oligosaccharides that selectively encourage the development and multiplication of microflora particularly *Lactobacilli* and *Bifidobacterium* in the gut. Characteristics of an ideal prebiotic include resistance against stomach acids, bile salts and enzymes, easily fermented and utilized by gut microflora. Breast milk, soy beans, raw oats, inulin sources, and unrefined barley and wheat are some sources of prebiotics. Inulin is considered as the predominant bifidogenic and non-digestible oligosaccharide which satisfies all the standards for prebiotic classification. Inulin when hydrolysed results in the production of Oligofructose and (trans) galacto-oligosaccharides (GOS). Prebiotics welfares host health by plummeting the occurrence and extent of diarrhea and helps in averting colon cancer. They also facilitate mineral uptake, lower the risk of heart diseases, and prevent obesity by encouraging weight loss (Anbukkarasi et al., 2014). Prebiotics and probiotics are the harmless and effective dietary substances, which can remedially alter the gut microbiota of the host and altogether are termed as synbiotics (Burn S.F, 2012). A synbiotic product aids the host by improving the survival and implantation of live microbial dietary supplements in the GIT by particularly encouraging the growth and/or activating the metabolism of health-promoting bacteria residing in the gut. According to US Department of Energy, the probiotic species *S. thermophilus* found in fermented dairy products produce lactase for breakdown of milk sugar lactose into glucose and galactose. This quality is beneficial for lactose intolerant (a condition also known as hypolactasia) people. After consumption of milk and/or dairy products, lactose intolerant individuals experience certain gastrointestinal symptoms due to shortage of adequate lactase (β-galactosidase) activity to effectively digest lactose resulting in bloating, diarrhea and abdominal pain. *S. thermophilus* is non-motile, fimbriated and does not produce endospores. The optimal growth temperature of this strain is from 35°C to 42 °C and is categorized as a lactic acid bacterium. It is widely used in food fermentations (Choksi N. and Desai H, 2012). It lacks genes containing surface proteins; therefore, the body's defense mechanism does not confuse it with harmful and pathogenic bacteria. The study of probiotics and prebiotics is important with regard to gastrointestinal disorders. Lactase producing probiotic bacteria are used in combination with prebiotic nutrients to overcome the problem of lactose intolerance. Lactase digests lactose in milk thus converting it into glucose and galactose. Inulin, a natural fructan, cannot be hydrolyzed by digestive enzymes in the human body and plays a role as a dietary fiber and prebiotic. Due to its versatile physicochemical properties and physiological functions, inulin has been widely applied in food,

pharmaceuticals, and many other fields (Dawei, N., 2019).

This research study focuses on how a probiotic bacterial strain produces a good amount of lactase enzyme in the presence of suitable prebiotic nutrient by determining the enzyme activity. This information can be useful for industry to develop probiotic and prebiotic formulations of certain food products and dietary supplements for lactose intolerant individuals.

2.Methodology

2.1 Sample Collection and Storage

Initially, yogurt and milk samples were collected from sweet meat shops in Lahore and Gujranwala to increase the possibility of isolating probiotic bacterial species for co-culture study. A mixed population of bacteria was obtained on nutrient agar. Selection of probiotic isolates was done on *Streptococcus thermophilus* Isolation Agar medium. 30 ml of each sample was collected in 50 ml sterilized falcon tubes. The sample containing falcon tubes were placed in an ice box, brought to Biotechnology Laboratory (Kinnaird College for Women) and stored at 4º C in a refrigerator.

2.2 Sterilization of Media and Apparatus

Media was prepared in conical flasks and plugged using cotton plugs and aluminum foil. All the apparatus to be used was first wrapped up in newspaper/Aluminum foil. Both the apparatus and media were sterilized in an autoclave for 15 minutes at 121º C and 15 psi prior to use. The ambience of the microbiological cabin was sterilized by burning spirit lamps for 15 minutes. The work bench top was cleaned using 70% ethanol as a disinfectant prior to start of experimental procedures.

2.3 Isolation of Bacterial Strains

2.3.1 Streak plate technique

Streak plate technique was used to isolate, purify and maintain the bacterial isolates. *Streptococcus thermophilus* Isolation Agar medium was prepared, sterilized and aseptically poured in to petri plates. The medium was allowed to solidify in petri plates at room temperature. The yogurt samples were used to inoculate the petri plates. For this step, an inoculating loop was sterilized by spraying it with 70% ethanol and flaming it over a spirit lamp until red hot. The loop was allowed to cool and then dipped in the sample and a loop full was streaked onto the petri plate. The plates were incubated at 42º C for 36-48 hrs. Two populations obtained after incubation were studied as a co-culture. These colonies were then used for identification and the preparation of fresh broth cultures required for biochemical tests.

2.4 Sub-culturing for obtaining Fresh Cultures

Batch cultures of bacterial isolates were prepared by inoculating them in Phenol Red Lactose Broth (PRLB). The co-culture was used in biochemical tests performed during the experimental study. The PRLB media was prepared, poured into conical flasks and sterilized by autoclaving it in an autoclave for 15 minutes. Under aseptic conditions, the bacterial cells were picked using an inoculating loop and transferred to the freshly prepared broth. The flasks were incubated in a shaking incubator at 120 rpm, 42ºC for 36-48 hrs. The flasks were later on used as per requirement. For sub-culturing, PRLB was prepared, poured in conical flasks and sterilized in an autoclave. 5 µl of 48 hour bacterial culture was taken using a micropipette and transferred into the flask containing freshly prepared PRLB. The flasks were then put on similar incubation conditions. Batch culturing was done every week to ensure the revival of culture.

2.5 White/Blue Screening Test

This screening test was used for the bacterial co-culture in order to make sure that the isolated strains produced lactase. 50 ml of Nutrient agar was prepared in a conical flask and autoclaved for 15 minutes. Under aseptic conditions, 50 µl of X-Gal was added to the medium and then medium was poured into autoclaved petri plates. The plates were set aside to let the medium solidify. The co-culture was aseptically streaked onto X-Gal infused nutrient media. The plates

were incubated at 42ºC for 36-48 hrs and later presence of blue and white colonies was observed. Presence of blue colonies showed the production of active lactase enzyme whereas white colonies showed otherwise.

2.6 Morphological Tests

2.6.1 Gram staining

A small amount of co-culture from the petri plate was mixed with a drop of distilled water on a clean glass slide. The slide was then air dried and heat fixed later. 4-5 drops of crystal violet were added to the smear for 1 minute. It was then gently rinsed with distilled water. 4-5 drops of Gram's iodine were added for one minute and rinsed gently with distilled water. Ethanol was added drop by drop to decolorize the smear, rinsed with distilled water and counterstained with safranin. After 45 seconds, the slide was washed with distilled water and viewed under 40X lens of the microscope. Spheres indicate *Cocci* whereas rods indicate *Bacilli*. Purple colored cells were regarded as Gram positive whereas pink colored cells were regarded as Gram positive.

2.7 Determination of Oxygen Requirement

Microbes have a diverse ability of using oxygen for respiration. On the basis of varying requirement of oxygen, microbes are classified into the following five categories: Obligate anaerobes, Aerotolerant anaerobes, Facultative anaerobes, Microaerophiles, and Aerobes. 5-7 ml of nutrient broth was added to a test tube and autoclaved for 15 minutes. The broth was then allowed to cool and 5 µl of 48 hour bacterial culture was inoculated in the test tube and shaken. The test tube was then incubated at 42ºC for 36-48 hours. The results were then observed.

2.8 Motility test

The motility test differentiates the microbes on the basis of their ability to be motile. 0.4% of the semi solid medium is prepared by dissolving 1.04g of nutrient broth and 0.4 g of nutrient agar in distilled water. The medium was poured into test tubes and then autoclaved for 15 minutes. X-Gal was also added to the media. The test tubes were allowed to cool in order for the media to solidify. An inoculating needle was sterilized over a flame and used to stab inoculate the medium with 48 hour culture.

2.9 Biochemical Tests

2.9.1 Catalase test

About 5µl of 48 hour bacterial co-culture was transferred to one of the two test tube containing sterile, freshly prepared nutrient broth. The test tubes were then incubated at 42 ºC for 36-48 hours. One test tube served as a control whereas 4-5 drops of 3% hydrogen peroxide were added to the sample test tube. Immediate bubbling shows a positive result indicating presence of catalase enzyme where no bubbling suggested otherwise.

2.9.2 Lactose utilization test

About 5µl of 48 hour bacterial co-culture was transferred to one of the two test tubes containing sterile, freshly prepared PRLB. The test tubes were then incubated at 42ºC for 36-48 hours. One of the test tubes served as control whereas the other contained sample. Change in the red colored broth to a yellow shade indicated towards positive result *i.e.* lowering of pH due to lactic acid production.

2.10 Preparation of Crude Enzyme

5 µl of bacterial co-culture was transferred to a conical flask containing fresh, sterile PRLB and incubated in a shaker incubator at 120 rpm and 42ºC for 36-48 hrs. After this incubation time, the medium containing the co-culture was transferred to 50, 1 ml sterilized Eppendorf vials, centrifuged at 12000 rpm for 20 minute. The pellet was discarded and the clear supernatant containing the crude enzyme was stored at 4ºC in a refrigerator.

2.11 Preparation of Organic Extracts

Inulin rich organic extracts of onion and garlic were prepared by separately blending 40 grams of onion and garlic in 80 ml of cold distilled water in a blender. The mixture was then filtered using Whatman filter paper to remove any solid components. The filtrate was then aseptically

transferred to sterile falcon tubes and centrifuged at 4000-6000 rpm for 20 minutes. Clear supernatant was stored at 4ºC in a refrigerator until further use.

2.12 Preparation of Glucose Standard Curve

Glucose was used as a standard reference curve for determining the lactase activity. For the preparation of the curve, 2% glucose solution was prepared in distilled water. Ten dilutions were made (0.2 -2.0%). DNS reagent (1 ml) was added and blank was prepared by adding 1ml of DNS and 1ml of distilled water. The test tubes after boiling for 5 minutes were gradually cooled. The contents of the test tube were diluted by adding 20 ml distilled water. Absorbance was taken at 540 nm. Graph was plotted for absorbance and glucose concentration.

2.13 Enzyme Assay

Three sets of five sterilized 100 ml conical flasks containing 49 ml Phenol Red lactose broth (PRLB) were taken, two of the flasks in each set served as positive and negative control, the rest three contained different concentrations of commercial inulin. Distilled water (1 ml) was used as negative control whereas, 48 hour bacterial co-culture (1 ml) was added without inulin for positive control. The commercial inulin concentrations used were, 0.2%, 0.4% and 0.8%. The same was repeated for aqueous extracts of both onion and garlic. The conical flasks were then incubated for 48 hours in a shaking incubator at 120 rpm and 42ºC. After incubation, 1ml of culture broth was taken from each flask in Eppendorf vials, centrifuged at 12000 rpm for 20 minutes. One ml of clear supernatant containing the crude enzyme was taken in a test tube with 1% lactose solution (1 ml) in 0.02 M sodium phosphate buffer at pH 7 (1ml). This was allowed to incubate at 25ºC for 10 minutes. Reaction between the enzyme and the substrate was stopped with 2 ml DNS. The reaction mix after boiling for 5 minutes was cooled to room temperature. This solution was further diluted with 20 ml distilled water to measure the absorbance at 540nm.

Calculation of enzyme units

The lactase activity was determined in IU/ml/min by applying the standard formula.

$$\text{Lactase activity (IU/ml/min)} = \frac{\text{Amount of sugar released} \times 1000}{\text{Molecular weight of Lactose} \times \text{Time of incubation}}$$

3.Results

A mixed population of bacteria was obtained on nutrient agar from yogurt and buffalo milk samples collected from sweet meat shops Lahore and Gujranwala. As a result of selection on *S. thermophilus* isolation agar medium, two probiotic bacterial isolates were obtained only from yogurt samples. No probiotic isolates could be found from milk.

3.1 Identification of Bacterial Isolates

3.1.1 Selective Media (M948)

The presence of sucrose in the *S. thermophilus* isolation medium allowed the growth of *S. thermophilus* and *L. bulgaricus,* one from each of the two yogurt samples.

3.1.2 Blue/White Screening Test

Both strains showed blue colonies when grown on nutrient agar infused with X-Gal. the blue color of the colony indicated the presence of lactase enzyme after X-gal hydrolysis (Figure 1).

3.1.3 Gram staining

Results of Gram's staining indicated that *S. thermophilus* was Gram positive, and stained as purple colored cocci while *L. bulgaricus* was also Gram positive and stained as purple rods.

3.1.4 Oxygen Requirement

Both the strains were facultative anaerobes as bacterial growth was observed throughout the medium because of their indifference to the absence or presence of oxygen.

3.1.5 Motility test

Both strains were observed to be non-motile as the growth was observed only along the line of stab inoculation.

3.2 Biochemical Tests

3.2.1 *S. thermophilus* and *L. bulgaricus* co-culture

Both strains were negative for catalase test on addition of hydrogen peroxide due to the absence of catalase enzyme. Growth on and color change of PRLB from red to yellow indicated the ability of the co-culture to ferment lactose.

3.3 Enzyme Assay

Effect of two organic inulin rich sources namely garlic and onion on the lactase activity of *S. thermophilus* and *L. bulgaricus* co-culture is described. The pH change from 6.6 to 3.5 was noticed in the co-culture at 42°C. Glucose standard curve (Figure 2) was used to calculate the amount of glucose by incorporating the absorbance values of glucose released from lactose hydrolysis in the reaction mixtures. The lactase enzyme activity is defined as the rate at which lactase converts lactose into glucose and galactose can be affected by initial glucose amount, pH and temperature.

Figures 3, 4, 5 show lactase activity and standard deviation in *Streptococcus* and *Lactobacillus* co-culture using 0.2%, 0.4%, 0.8% concentration of aqueous garlic extracts, aqueous onion extracts and commercial inulin. Enzyme activity obtained with garlic and onion extracts was 3.21, 4.03, 4.29 IU/ml/min and 3.97, 3.91, 4.03 IU/ml/min with standard deviation of 2.436+0.199, 2.799+0.091, 2.945+0.026, and 2.649+0.130, 2.656+0.083, 2.672+0.081, respectively. Enzyme activity with commercial inulin used as a positive control was 4.38, 4.32, 3.80 IU/ml/min resulting in standard deviation of 3.030+0.161, 2.949+0.113, 2.860+0.024.

4. Discussion

S. thermophilus Isolation Agar is used to determine the proportion of *S. thermophilus* and *L. bulgaricus* in yoghurt. These two strains are present in a symbiotic relationship in yogurt. For prime consistency, taste and aroma in yogurt the two species must be present in equal numbers as co-culture. The formulation of *S. thermophilus* Isolation Agar was developed by Lee S. Y. et al. (1974) to enumerate *S. thermophilus* and *L. bulgaricus*. *S.thermophilus* utilizes sucrose present in the medium. Lactose present in the medium is exploited by both the strains. With a suitable combination of sucrose and lactose, the rate of acid production by *S. thermophilus* is enhanced. Casein hydrolysate (enzymic) and yeast extract provide nitrogen, vitamin B and trace elements for the growth of *S. thermophilus*. Dipotassium phosphate maintains pH in the medium.

In blue/white screening test, the bacterial strains were streaked on nutrient broth infused with X-gal. The presence of blue colonies indicated the activity of β-galactosidase which catalyzes the breakdown of lactose into monosaccharide galactose and glucose. As reported by Burn S.F. (2012) hydrolysis causes the splitting of lactose molecule by cleaving the oxygen bridge. β-galactosidase enzyme hydrolyzes X-gal, analog of lactose into galactose and 5-bromo-4-chloro-3-hydroxyindole. This is oxidized into 5, 5'-dibromo-4, 4'-dichloro-indigo, an insoluble blue product which indicates β-galactosidase activity.

Biochemical tests are vital in order to confirm specific characteristics about the identified strains. One of the biochemical tests includes the color change of PRLB. It is used for detection of lactose fermenting bacteria. Phenol Red Broth Medium prepared according to Vera H.D. (1950) is used to ascertain the fermentation reaction of carbohydrates for the differentiation of microbes. Proteose peptone and beef extract act as sources of carbon and nitrogen. Sodium chloride is used to achieve osmotic stabilization. Phenol red is the pH indicator, which changes to yellow at acidic pH on fermentation of lactose. In addition to producing a pH color shift, the creation of mixed acids, remarkably butyric

acids, results in a pungent, foul odor from the culture medium.

With increasing concentration of garlic extract the concentration of glucose was increased as indicated by the absorbance value for aqueous garlic extracts. The lactase activity in concentrations 0.2%, 0.4% and 0.8% was 3.21 IU/ml/min, 4.03 IU/ml/min and 4.29 IU/ml/min respectively. This data indicates that increase in the concentration of inulin rich garlic extract is proportional to the glucose released in the reaction mixture due to the action of lactase enzyme.

Different concentrations of aqueous onion extract were observed to have effect on the enzyme activity which increased gradually from 3.97 IU/ml/min to 3.91 IU/ml/min and 4.035 IU/ml/min for concentrations 0.2%, 0.4% and 0.8% of onion extract. Once the concentration is reduced, this affects the lactase enzyme activity and production of glucose.

Commercial inulin used as a prebiotic has given maximum enzyme activity at minimum concentration of 0.2% (4.38 IU/ml/min) as compared to glucose values obtained with the same concentration of onion and garlic extracts. This difference might be due to the fact that in the extracts, inulin alone is not present; there are other substances that might have reduced lactase activity thus affecting the hydrolysis of lactose into glucose to some extent. Garlic extracts (0.4%, 0.8%) have shown higher enzyme activity as compared to onions extracts at the same concentrations.

Results of a study by Ricardo P.S.O. (2012) highlighted stimulation of the overall metabolism of Lactic acid bacteria induced by inulin, as the levels of all metabolic end products increased. The increased levels of ethanol and acetic acid in both mono-culture and co-culture of Space-Time Low-Rate (St-Lr) suggest that some fructose released from partial inulin hydrolysis was hetero fermented by *Lactobacillus* monoculture.

In the present study, bacterial co-culture induced by inulin rich onion and garlic extracts has shown increased lactase activity in comparison with commercial inulin used as the positive control.

The addition of prebiotic inulin has a significant effect on probiotics such as *Lactobacillus spp* and this combination with inulin could be a viable probiotic-based functional food approach or treating *E.coli* related infections according to findings of Derya et al. (2018).

The results of the study suggest that onion and garlic extracts can be further studied and used for extraction and purification of inulin for commercialization with reference to healthy probiotic food products.

5.Conclusion

The two probiotic strains have been identified as *Streptococcus* and *Lactobacillus* from yogurt. Inulin rich garlic extracts with 0.4% and 0.8% concentrations are a better choice for lactase production in a co-culture of the two strains as compared to onion extracts. However, this enzyme activity is less than what is obtained with commercial inulin used in the study.

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Figure 1: Lactase producing co-culture on Nutrient Agar infused with X-gal.

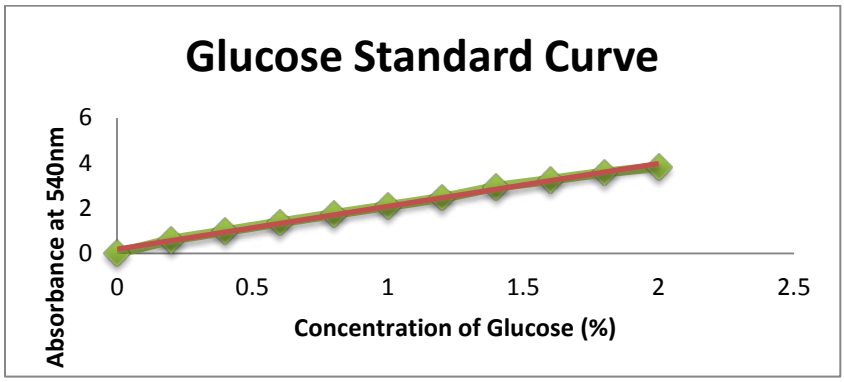

Figure 2: Standard calibration curve of gluscose.

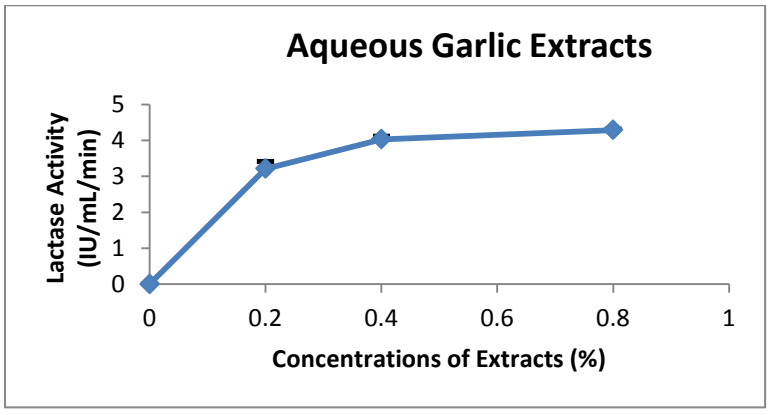

Figure 3: Lactase activity with different concentrations of garlic extract.

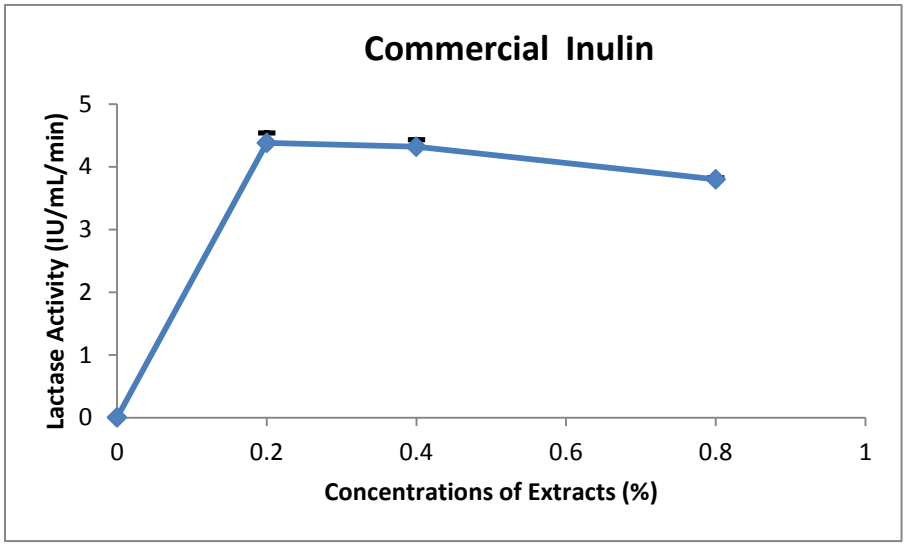

Figure 5: Lactase activity with different concentrations of commercial inulin.

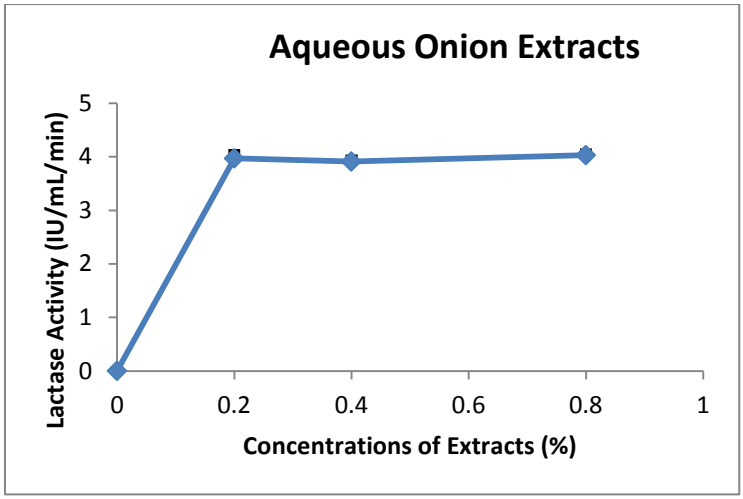

Figure 4: Lactase activity with different concentrations of onion extract.