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COMPARATIVE STUDY OF IMMOBILIZED ENZYME AND FREE ENZYME OF PARTIALLY PURIFIED BETA AMYLASE ISOLATED FROM SWEET POTATO (*IPOMOEA BATATAS)*

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ABSTRACT

Exoamylase / β-amylase hydrolyzes polyglucan chains'α-1,4glycosidic connections at the non-reducing end to create maltose (4-O-α-d-Glucopyranosyl-β-d-Glc). Maltose's lowering Glc is present in the β-form. The primary function of *β*-amylase is involvement in starch breakdown in plants. Aim of the present study was to make immobilized beta amylase enzyme using natural matrix by entrapment method under optimum temperature and pH. Then compare the immobilization of beta-amylase which is extracted from *Ipomoea batatas* (sweet potato) with free enzyme (partial purification). Extraction was performed by ammonium sulfate precipitation method whereas partial purification was finished by dialysis bags. Then performed starch hydrolysis confirmatory test using iodine solution. The partially purified enzyme was immobilized through 4% of sodium alginate and agar dissolved in 0.1M phosphate buffer in addition of 0.4M $CaCl_2$ respectively. The molecular weight of enzyme was determined to be 8KDa through PAGE electrophoresis. After immobilization of enzyme, its stability was checked on weekly basis. Hence all comparatively study demonstrated that immobilization of beta amylase produced with sodium alginate show relatively great properties that can be applicable in various fields
Keywords: Beta amylase, Immobilization, Partial purification, electrophoresis, Sodium alginate.

Introduction

The hydrolase class of enzymes includes a sizable group called amylase, comprising 30 members. Within this group, the hydrolytic nature is exhibited by two enzymes known as α and β-amylases. α-amylases break down starch into maltose, glucose, and maltodextrin, while β-amylases hydrolyze the non-reducing chain end of starch, yielding specific enzymes such as maltose and dextrin (Souza and Magalhaes, 2010). β-amylase, also known as α-1,4-glucan maltohydrolase, is a highly prevalent and thermostable enzyme that is renowned for its ability to break the α-1,4-glycosidic bond at the non-reducing end of starch, ultimately yielding maltose and maltodextrins. The glucose unit in the reducing end of maltose is in the β-form, hence the name β-amylase (Xuguo Duan *et al*., 2021; Kushwaha *et al*., 2021). As the industrial applications of β-amylase are being evaluated, its production is currently increasing (Kossmann and Lloyd, 2000). Sweet potatoes, soybeans and barley are extensively utilized as sources for producing β-amylase in industrial sectors (Das *et al*., 2018 Kushwaha *et al*., 2020). The role of sweet potatoes in the global effort to combat vitamin A deficiency (VAD) is significant (Schweigert *et al*., 2003; Stephenson *et al*., 2000; Underwood and Arthur, 1996).

Functions of β–amylase

β-amylase plays a crucial role in breaking down starch, facilitating seed germination and fruit ripening, as well as contributing to their sweet taste. Starch, found in granules, is the primary form of energy storage in plants. It comprises two glucose polymers, amylose and amylopectin, which are linked by glycosidic bonds. These constituents of starch possess distinct structures and properties (Figure 1). Extensive research has been conducted on the involvement of β-amylase in hydrolyzing starch reserves in cereal endosperm, promoting germination under in vivo conditions. Additionally, various plant tissues, including leaves, have been found to possess high levels of β-amylase activity. For instance, in pea and *Arabidopsis*, β-amylase activity exceeds that of other glucan-metabolizing enzymes by multiple folds (Wu *et al*., 2011). β-amylases predominantly produce maltose as the primary product during starch degradation, while glucose is a minor product. Both glucose and maltose are transported out of the chloroplast through specific transporters (Zeeman *et al*., 2004).

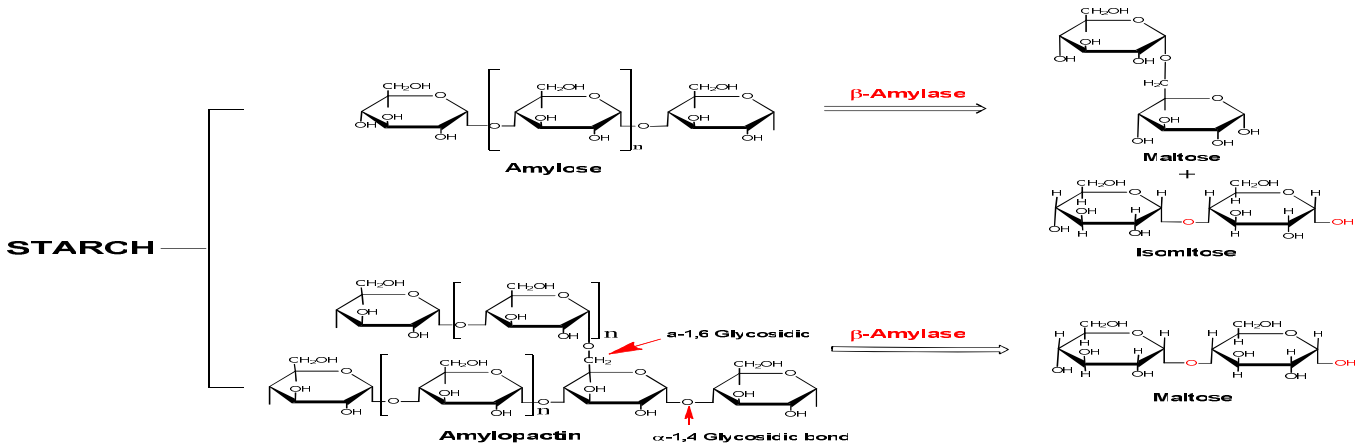

Fig. 1: β-amylase catalyzes the hydrolysis of starch, yielding amylose and amylopectin as intermediate products, which are subsequently broken down into maltose and isomaltose

Immobilization of enzymes

Immobilization of enzyme is widely distributed technology to fixing an active enzyme for reuse. An immobilized providing more stability of active enzymes to prevent against in activators. The immobilization of enzyme, enzymatic reaction are highly beneficial. Therefore during enzyme-substrate reactions, some time the resultant product gets contaminated or it leads to the formation of undesirable product. In this case, immobilization of enzymes is an efficient method to separate the complete enzyme from the product and also separate the undesirable proteins from the product (Kallenberg *et al*., 2005).

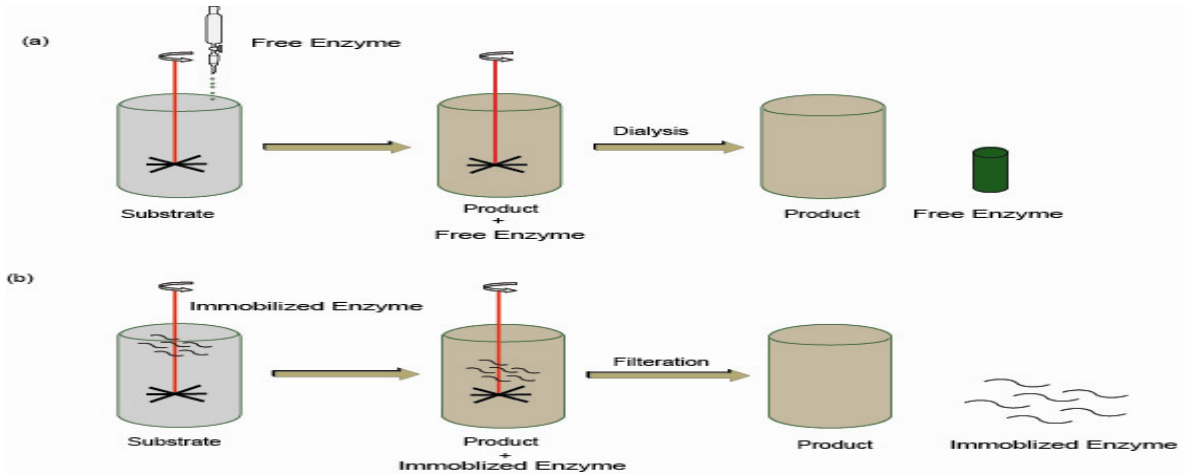

Fig. 2 : Immobilization of enzyme

Factors responsible for enzyme immobilization

Enzyme characteristics such as tolerance to immobilisation, chemical and physical environment, surface functional groups, size, charge, polarity, and transport requirements influence the methods used for enzyme immobilisation (Hanefeld *et al*., 2009). Enzyme immobilisation techniques include adsorption, cross-linking, entrapment/encapsulation, and physical entrapment inside a polymeric matrix (Lim *et al*., 2007). Entrapment involves polymerizing a monomer or low molecular weight polymer around a protein to trap it on a surface. This approach is commonly used with hydrogels and sol-gels to ensure the protein cannot diffuse out of the polymers. Entrapment is a well-established technique for encapsulating enzymes and other functional proteins based on sol-gel chemistry (Hussain *et al*., 2005).

Application of Enzyme immobilization

Immobilized enzymes have numerous applications in industry, medicine, food and research, including biosensors, digestive enzyme immobilization, textile and paper industries. The main sources of β-amylase are wheat, sweet potatoes, and cereal grains, with sweet potatoes having the highest concentration of the enzyme. β-amylase is utilized in the production of maltose syrup and has potential medicinal uses due to its antioxidant, antibacterial, anti-inflammatory and antihypertensive properties. Sweet potatoes are a cost-effective and abundant source of β-amylase and can be directly immobilized from raw homogenates, eliminating the need for enzyme purification. Immobilized β-amylase has advantages over free enzymes, including increased thermostability, reduced bacterial contamination, shorter reaction times, and lower substrate and product viscosity.

Material and Methods

2.1 Extraction of crude enzyme from sweet potatoes

The skin was removed from a sweet potato weighing about 100 g. They were cut into small pieces, combined with 16 ml of cool 20 mM sodium phosphate buffer (pH 6), and mixed for three minutes. Before being placed in centrifuge tubes, the ground material was screened through muslin cloth. After that, the filtrate underwent a 20-minute centrifugation process at 4°C and 12,000 rpm. Following centrifugation, the supernatant was collected. The

supernatant underwent protein estimation through (Kushwaha and Verma, 2017).

2.2 Partially purifying a raw enzyme

Freshly made ammonium sulphate (0.47 g/ml) was combined with the extracted supernatant solution and the mixture was left overnight. The following day, the same solution underwent a second centrifugation at 12,000 rpm for 20 minutes to separate the supernatant. Pellets were dissolved in a 20 mM phosphate solution. The pellets were kept in a dialysis bag and dissolved in a 20 mM, pH 6 sodium phosphate buffer for dialyzing against pure water. The loaded dialysis bag was immersed in a sucrose solution for an hour to concentrate the enzyme sample. The enzyme was then taken out of the dialysis bag and stored for potential future enzyme activity analysis by Olaniyi *et al*., 2010.

2.3 Confirmatory test for starch hydrolysis

After an overnight incubation on a starch-agar plate, the presence of a clear zone of hydrolysis will indicate that the enzyme has undergone some purification.

2.4 characterization beta amylase *via* electrophoresis

The partly pure beta amylase was electrophoresed on a 12% polyacrylamide gel using the biolab protein marker (2-212 kDa) developed by Laemmli in 1970.

2.5 Using two different natural matrices, beta amylase was immobilised by the entrapment method:

2.5.1 Sodium alginate is used to immobilize: The enzyme was immobilised using a technique involving sodium alginate. By heating the components to 50 °C, a fresh 4% sodium alginate solution was created in a 0.1 M phosphate buffer with a pH of 7. After cooling to room temperature, 1 ml of the enzyme stock solution and 9 ml of the sodium alginate solution were mixed (the enzyme and sodium alginate solution ratio was 1:9, and the total volume of the matrix and enzyme mixture was 10 ml). The mixture was then suspended dropwise into a cool 0.4 M calcium chloride solution and gently swirled for two hours at 4°C. Beads were successfully recovered and easily produced through filtration. The finished beads were thoroughly cleaned multiple times with distilled water before being stored at 4 °C in 0.1 M phosphate buffer (pH 7.0) for additional enzyme immobilisation testing. An enzyme activity test was performed on the calcium chloride solution filtrate determination by Rajagopalan and Krishnan, 2008.

2.5.2 Agar-based immobilisation: The beta-amylase enzyme was immobilised using a different immobilisation method that used agar as the matrix. Using this method, a fresh 4% solution of agar was created in a 0.1 M phosphate buffer with a pH of 7.0 by heating the elements at 50 °C. The mixture, which contained a 1:9 ratio of enzyme to agar solution and a total volume of 10 ml, was cast onto prefabricated glass plates as soon as it reached room temperature. The gel was split into tiny pieces after it had set at room temperature and washed repeatedly in distilled water to remove any enzyme that had attached itself to the gel surface. The beads were stored in a pH 7.0, 0.1 M phosphate buffer at 4 °C. (Matsunga *et al*., 1980. Katiyar *et al.,* 2018) measured the enzyme activity in both free and immobilised forms and protein estimation was done through (Kushwaha and Verma, 2017).

Results

Extraction of partial purification of Beta-amylase enzyme

Extraction of beta-amylase was done by sweet potato which is a rich source of this enzyme, later on purification of this enzyme was done using ammonium sulphate precipitation method. The enzyme solution was precipitated at 45% saturation. Further dialysis was done in order to obtain partially purified enzyme. Crude beta-amylase was derived from sweet potatoes (*Ipomoea batatas*) showed specific activity 7.83U/mg whereas the purification fold was one. Extraction of crude enzyme from sweet potato showed 100% yield and after centrifugation specific activity increased to 9.23U/mg. 80.09% yield and purification fold of 1.17. Starting from extraction of crude enzyme to protein dialysis, enzyme activity declined sand specific activity increased

Dialysis of Beta-amylase after ammonium sulphate precipitation

After ammonium sulphate precipitation, the salt was removed in beta–amylase through dialysis process. Starting from extraction of crude enzyme to protein dialysis, enzyme activity declined and specific activity increased. Specific activity of ammonium sulphate precipitation and protein dialysis was 15.11U/mg and 22.7U/mg ammonium sulphate precipitation yield showed 62% its purification fold 1.9 observed. Protein dialysis purification fold was 2.8 and yield 46.23% as shown in (Table 1).

Starch hydrolysis confirmatory test

Partial purification of beta amylase enzyme was confirmed by using starch hydrolysis test in which purified enzyme was incubated overnight on starch agar plate. After adding iodine solution, the clear hydrolysis zone on the agar plate was visible, indicating the presence of the enzyme. Fig. A and B demonstrate the plate's zone of clearance (Fig 3).

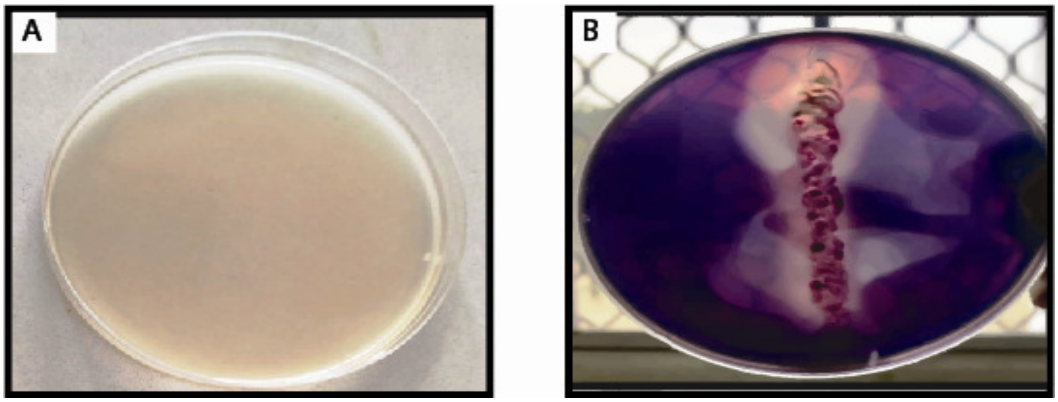

Fig. 3: Starch hydrolysis of partial purified beta amylase enzyme

Electrophoretic analysis of Beta-amylase

After obtaining the beta-amylase enzyme through dialysis, it was partially purified and its molecular weight was determined by using 12% Polyacrylamide Gel Electrophoresis (PAGE) and comparable standard protein markers. Purified beta amylase estimated molecular weight was 8 kDa, (Fig 4). SDS-PAGE electrophoresis studies revealed that molecular weight of β-amylase was 8kDa and partially purified β-amylase using ammonium sulphate fractionation showed 62% recovery. After protein dialysis 46.23% enzyme yield was obtained.

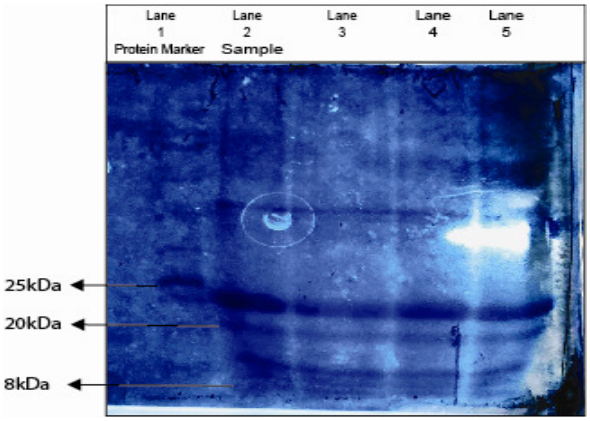

Fig. 4: Electrophoretic analysis of Beta-amylase

Immobilization by using Sodium alginate as matrix

Sodium alginate is a nontoxic, highly stable, highly porous and economical supporting matrix popularly used in enzyme immobilization techniques. In the present study immobilization of beta-amylase was first done by using 4% sodium alginate (Fig. 5). The results obtained were as follows: 273.059 µmol/min enzyme activity with 55.60% yield, total protein content 7.56 mg, amount of protein 0.28 mg. Specific activity and It was discovered that the overall beta amylase activity was 36.11 U/mg and 7372.59 respectively presented in table.1.

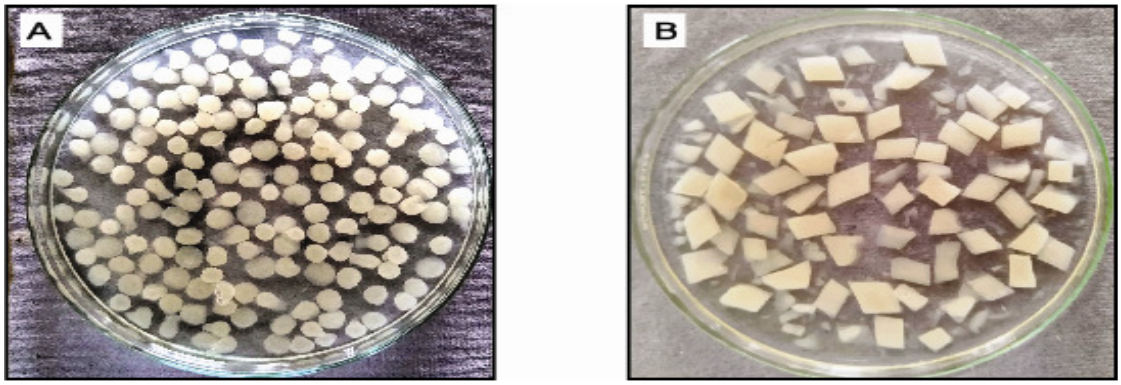

Fig. 5: Matrix beads formation for immobilization of beta amylase: (A) 4% Sodium alginate beads (B) 4% Agar beads.

Table 1: Purification of Beta-amylase

SN.		Method	Volume (ml)	Enzyme activity (µmol/min)	Amt. of Protein (mg/ml)	Total protein in fixed volume (mg)	Specific activity (U/mg)	Total activity (µmol/ min)	Purification fold	Yield (100%)
1	Free Enzyme (Partial Purification)	Extraction	60	220.989	0.47	28.2	7.83	13259.34	1	100
2.		After centrifugation	50	212.39	0.46	23	9.23	10619.5	1.17	80.09
3.		Ammonium sulphate precipitation	40	205.54	0.34	13.6	15.11	8221.6	1.9	62
4.		Protein dialysis	30	204.34	0.30	9	22.7	6130.2	2.8	46.23
5.	Immobilized Enzyme	Sodium Aliginate Immobilization	27	273.059	0.28	7.56	36.11	7372.59	4.6	55.60
6.		Agar Immobilization	26	234.584	0.25	6.5	36.08	6099.18	4.6	45.99
1	Free Enzyme (Partial Purification)	Extraction	60	220.989	0.47	28.2	7.83	13259.34	1	100
2.		After centrifugation	50	212.39	0.46	23	9.23	10619.5	1.17	80.09
3.		Ammonium sulphate precipitation	40	205.54	0.34	13.6	15.11	8221.6	1.9	62
4.		Protein dialysis	30	204.34	0.30	9	22.7	6130.2	2.8	46.23
5.	Immobilized Enzyme	Sodium Aliginate Immobilization	27	273.059	0.28	7.56	36.11	7372.59	4.6	55.60
6.		Agar Immobilization	26	234.584	0.25	6.5	36.08	6099.18	4.6	45.99

Stability of Sodium alginate immobilization

When sodium alginate was used as enzyme immobilizing matrix the enzyme activity lasted till 30 days and after 30 days the enzyme got denatured. The enzyme during this time period was recycled 5 times as shown in table 2. Sodium alginate mediated immobilization of beta amylase showed more stability when compared with free enzyme (partially purified), as yield percentage with sodium alginate was 55.60% as compared to free enzyme which was 46.23%. Hence all comparative studies demonstrated that immobilization of beta amylase on sodium alginate showed relatively better properties that can be applicable in various field. Sodium alginate immobilized β-amylase showed highest specific activity 36.11 U/mg comparing with agar immobilized β-amylase which is 36.08 U/mg. In the sequential studies it was found that sodium alginate showed highest purification fold of 4.6. Based on the ongoing analysis, it can be predicted that sweet potato extracted and sodium alginate immobilized β-amylase has potential to be employed for food as well as other industrial purposes.

Table 2 : Enzyme stability when Sodium alginate was used as matrix

Weekly recycled enzyme	Volume (ml)	Enzyme activity (µmol/mi)	Amt. of protein (mg/ml)	Total protein in fixed volume (mg)	Specific activity (U/mg)	Total activity (µmol/mi)	Purification fold	Yield (100%)
Week 1	24	223.163	0.27	6.4	34.86	5355.912	4.4	40.39
Week 2	21	192.383	0.23	4.8	40.07	4040.043	5.1	30.46
Week 3	17	147.453	0.20	3.4	43.3	2506.701	5.5	18.9
Week 4	14	70.995	0.12	1.6	44.3	993.93	5.6	7
Week 5	11	49.64	0.06	0.6	82.7	546.04	10.6	0.04

Stability of agar immobilization

When agar was used as enzyme immobilizing matrix the enzyme activity lasted till 21 days and after 21days the enzyme got denatured. The enzyme during this time period was recycled 5 times as shown in table 3

Table 3: Enzyme stability when agar was used as matrix

Weekly recycled enzyme	Volume (ml)	Enzyme activity (µmol/mi)	Amt. of protein (mg/ml)	Total protein in fixed volume (mg)	Specific activity (U/mg)	Total activity (µmol/mi)	Purification fold	Yield (100%)
Week 1	24	222.171	0.28	6.7	33.15	5332.104	4.2	40.2
Week 2	21	184.935	0.24	5	36.98	3883.635	4.7	29.2
Week 3	17	139.756	0.18	3	46.58	2375.852	5.9	17.9
Week 4	14	65.284	0.10	1.4	46.63	913.976	5.9	0.06
Week 5	11	26.06	0.05	0.5	52.12	286.66	6.6	0.02

Discussion

The current study compares immobilised enzyme with selectivity of extraction and partial purification of β -amylase from sweet potatoes. Ammonium sulphate was used in the traditional salt precipitation process. Sweet potatoes were discovered to be an inexpensive source with a high amount of the enzyme amylase (Dwivedi *et al*., 2021). Beta-carotene, calcium, potassium, and vitamins A and C are all present in sweet potatoes (Dutta *et al*., 2014). In comparison to free enzyme, immobilised amylases are more stable. the key benefits of employing sodium alginate matrix include its non-toxicity, high stability, high porosity, straightforward immobilisation technique and relatively low cost at laboratory scale .

The beads' pores should be large enough for substrate and product to permeate easily into and out of the alginate gel matrix, while the enzyme should remain contained inside the microenvironment of the beads.as suggested by Riaz and Coworkers (2009). Beyond this, the activity declines due to structure unfolding transition at high temperature and pH (Kushwaha *et al*., 2020). In the present study partially purified immobilized β-amylase after ammonium sulphate fractionation showed 62% recovery. After protein dialysis enzyme exhibited 46.23% yield and was further immobilised using the entrapment method on several matrices, including agar and alginate. The yield of β-amylase immobilised on sodium alginate was 55.60%. As observed by Anwar *et al.,* (2009) and Riaz *et al.* (2009) when compared with agar which was 45.99%. Amylase immobilized on alginate matrix was found to better with maximum immobilization yield 55.60% as reported by Prakash and Jaiswal (2011) and Sharma *et al.* (2014). As described by De Schepper *et al.* (2022), there is dynamic conversion of starch to fermentable sugars and dextrin during brewing process. While avoiding thermal inactivation of amylase in average time period, starch gelatinization and enzyme activity was enhanced focusing throughout on thermostable properties of barley malt's α and β-amylases. In their current studies, first order reaction for α-amylase was observed whereas for β-amylase, it was found that 13% of β-amylase degraded on extensive heating at 72.5°C. Majority of β-amylase enzyme showed thermolabile and thermostable characters. By using small scale mashing process, their residual activities were predicted.

Conclusion

This study extracted crude β-amylase from sweet potatoes and used various purification techniques such as centrifugation, ammonium sulfate precipitation, and protein dialysis. The partially purified β-amylase was immobilized using two matrices, sodium alginate and agar, and compared for their yield and stability. The sodium alginate-immobilized β-amylase showed a maximum yield of 55.60% and the

highest specific activity of 36.11 U/mg. It also had a higher purification fold of 4.6 and was found to be more stable than the free enzyme. The immobilized enzyme was stable up to 30 days. The study concluded that immobilized β-amylase on sodium alginate is more efficient and stable and can be used in various industries such as food, fermentation, textile, and pharmaceuticals.

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