Journal of Materials and Environmental Sciences
ISSN : 2028-2508
CODEN : JMESCN

J. Mater. Environ. Sci., 2018, Volume 9 , Issue 8, Page 2315-2321

http://www.jmaterenvironsci.com

Immobilization of Inulinase Produced by *Rhizopus oligosporus* NRRL 2549 for continuous fructose production

E. A. Karam [1] , M. E. Hassan [2], M. E. Moharam [1] and A. L. Kansoh [1]*.

[1]*Microbial Chemistry Dept National Research Centre, .Dokki, Giza, Cairo, Egypt.*
[2]*Centre of Excellence, Encapsulation Nanobiotechnology Group. Natural and Microbial Product Chemistry Dept National Research Centre, .Dokki, Giza, Cairo, Egypt.*

Received 07 Dec 2017,
Revised 20 May 2018,
Accepted 26 May 2018

Keywords
- ✓ Inulinase ,
- ✓ Enzyme,
- ✓ Stability,
- ✓ *Rhizopus oligosporus*,
- ✓ Immobilization,

*amany_kansoh@yahoo.com

Abstract

Crude inulinase produced by *Rhizopus oligosporus* NRRL2549 was effectively immobilized on a novel alginate/k-Carrageen beads that were hardened with glutaraldehyde. The pH activities of the free and immobilized enzyme were optimal at pHs of 5.5 and 6.0, respectively. The optimal reaction temperature was shifted from 45°C to 55°C for the immobilized inulinase enzyme. On the other hand, thermal stability investigated that the activity of the free enzyme was reduced to 54% at 80˚C after 30 minute while the immobilized form retained 76% of its activity at the same condition. Achieved immobilization yield was 84.6 % and the immobilized enzyme was reused 18 times retaining 90.2% of its activity. continuous production of fructose from inulin by the immobilized inulinase was also investigated.

1. Introduction

Inulinases that degrade the β-[2, 1] linkages of β-fructans, like inulin are classified into endo- and exo-inulinases, depending on their mode of action. "Endo-inulinases [2,1-β-D-fructanfructanohydrolase ; EC 3.2.1] are specific for inulin and hydrolyse it by breaking bonds between fructose units that are located away from the ends of the polymer network, to produce oligosaccharides [1]. Exo-inulinases [β-D-fructohydrolase; EC 3.2.1], split terminal fructose units in sucrose, raffinose and inulin to liberate fructose" [2,3,4].

The food industry is a field where biotechnology finds a wide application. Within this context fructose have potential applications in many industrial steps, including "food processing, pharmaceutical and medical industries" [5], it is consider "safe alternative sweetener to sucrose" [6].

Inulinases are encountered "in plants" [7], filamentous fungi, yeasts, and bacteria. Among the fungi,"*Aspergillus* spp". [8,9,10,11], "*Penicillium* spp.", [12,13] and filamentous fungus," *Rhizopus* sp. strain TN-96 "[4]. Yeasts are common inulinase-producers.," *Kluyveromyces marxianus"* [14,15] and *"Saccharomyces cerevisiae"* [16]. Also, bacteria are good producers of inulinase as "*Bacillus* spp., *Clostridium* spp. and *Xanthomonas* spp." [17].

Calcium alginate hydrogel beads are commonly used carriers in the entrapment immobilization enzymes [18] that is due to their significant advantages such as high porosity, low cost , and simplicity of preparation, but this material has some limitations due to biocompatibility, including high bimolecular leakage, and large pore size. For control release of the enzyme from the gel matrix and encapsulation efficiency, the covalent cross-linking with polymers, such as Carrageen ,chitosan, and coating the surface of alginate gel beads with other reagents such as" glutaraldehyde", were used [19].

Immobilization extends the stability of the enzyme by protecting the active material from deactivation and enables repeated use. Also, it provides significant reduction in the operation costs, facilitates easy separation of the enzyme and speeds up its recovery.

The aim of this work is the immobilization of inulinase produced from *Rhizopus oligosporus* NRRL 2549 on Sodium alginate/k-Carrageenan gel as a support, and studying some properties of the immobilized

inulinase enzyme compared with the free one. Also, continuous production of fructose from inulin by the immobilized inulinase was investigated in a packed-bed column reactor.

2. Material and Methods

2.1 Microorganism

R. oligosporus NRRL 2549 was obtained from the culture collection of the Northern Regional Research Laboratory, USDA, Peoria, IL, USA.

2.2 Medium and growth conditions

The organism was grown on 5% finely ground dry artichoke leaves in water as a complete growth medium [pH 7.0] with a particle size of less than 300 micrometers. Incubation was carried out at 30 °C for 48 h. in a rotary shaker operated at 200 rpm. After centrifugation at 4000 rpm for 20 min, the supernatant was collected as the crude enzyme extract.

2.3 Inulinase assays

The activities of both free and immobilized inulinase were determined by measuring the amount of fructose liberated using inulin as substrate by DNS method [20]. The reaction mixture [1ml] containing 0.5 ml of 4% [w/v] inulin in 0.05 M sodium acetate buffer, pH 5.5 and 0.5 ml of the culture filtrate, was incubated at 40 ºC for 20 min. the reaction was stopped by addition of 1 ml DNS , thin boiled for 5 min. after dilution , the optical density was measured at 540 nm.

One unit of inulinase was defined as the amount of enzyme that produce one micromole of fructose per min under the experimental assay conditions.

2.4 Preparation of Sodium alginate/k-Carrageenan beads

Sodium alginate/k-Carrageenan gel [ALG. CAR] was prepared by dissolving sodium alginate/k-carragenan [1:1 w:w] in distilled water to get a final concentration of 2% [w/v] alginate/carragenan gel. The gel solution was mixed thoroughly using an overhead mechanical stirrer until complete dissolving had occurred. The polymer solution was dropped into calcium chloride [$CaCl_2$] by using Encapsulator with nozzle size 300 µm to form uniform gel beads. The alg.Car.gel beads were hardened using $CaCl_2$ for 3 h. To modify the gel beads for covalent immobilization of enzyme, the alg.-Car. gel beads were putted into a solution of 4% [v/v] polyethylenimine [PEI] at pH 9.5 for 3 hrs. after washing with distilled water it soaked in glutaraldehyde [GA] 2.5 % for 3 hrs. and after washing , the beads was ready for immobilization.

2.5 Immobilization technique

0.5 g from Sodium alginate/k-Carrageenan [ALG. CAR] bead was inserted into 0.5 ml of culture filtrate [17.2 U at 4˚C for 24 h . Then, the unbound enzyme was removed by washing with distilled water until no protein or activity was detected in the wash.

2.6 Operational Stability

It was performed with 0.5g of the wet immobilized inulinase. The immobilized form was incubated with 1ml of 4% [w/v] inulin in 0.05 M sodium acetate buffer, pH 6.0 at 40°C for 20 min. At the end of the reaction period, it was collected, washed with distilled water and resuspended in 1 ml of freshly prepared substrate to start a new run. The supernatant was assayed for inulinase activity.

2.7 Effect of pH Value of free and immobilized enzyme

The effect of pHs on the free and immobilized enzyme activities were investigated in 0.1 M citrate phosphate buffer [pH 3.0 - 7.0], 0.1 M phosphate buffer [pH 7.0 - 8.0] and 0.1 M Tris - HCL buffer [pH 8.0 - 9.0], using the experimental assay conditions.

2.8 Effect of different temperatures of free and immobilized enzyme

Identical reaction mixtures were incubated at different temperatures [30 -80˚C] for 20 min. at pH 5.5 and pH 0.6 for free and immobilized enzyme, respectively. The enzyme assay was done as discussed previously.

2.9 Thermal stability of free and immobilized enzyme

The thermal stabilities of the free and immobilized inulinase were determined by measuring the residual enzyme activity exposed to different temperatures [40–80 ºC] for 2 h. and tested at 30 min time interval.

2.10 Immobilization yield

Immobilization yield [U/g carrier] was calculated according to the following equation: Immobilization yield [%] = Immobilized enzyme activity / Enzyme added activity − Unbound enzyme activity × 100.

2.11 Continuous inulin hydrolysis by immobilized inulinase

Continuous hydrolysis of inulin were performed in a water-jacketed packed bed column reactor of 1.5 cm diameter and 30 cm long. The assembled column was packed with 25 g of the immobilized inulinase to10 cm in height. The packed column was maintained at desired temperature by circulating water through the jacket. inulin solution in 0.05 M sodium acetate buffer, pH 5.5 were fed continuously into column downward flow using a peristaltic pump [21].

2.12 Scanning Electron Microscope [SEM] for alginate / carrageenan gel beads

Surface of different gel formulations, alginate/carragenan [Alg.car.] , Alg.car./ polyethylenimin [Alg.car./PEI] and Alg.car./PEI/ glutaraldehyde [GA] enzyme were examined using scanning electron microscopy [SEM, S-590, HITACHI] to prove the changes that occurred on surface after each reaction.

3. Results and discussion

3.1 Immobilization of inulinase enzyme

Fig. 1 shows the immobilization yield of the extracellular inulinase activity from *R. oligosporus* NRRL 2549 on three cheap carriers [Sodium alginate , K-Carrageenan and Sodium alginate/k-Carrageenan]. The highest immobilization yield [84.6%] was on Sodium alginate/k-Carrageenan carrier, that was chosen for studying the properties of the immobilized enzyme. *R. oligosporus* NRRL 2549 was a good producer of extracellular inulinase by submerged fermentation used artichoke leaves as sole nutrient and this result was coincided with [22]. Carrageenans are commercially important hydrophilic colloids, Chemically they are highly sulfated galactans . Their half-ester sulfate moieties making it strongly anionic polymers. "Alginate, are anionic polymers consist of mannuronic and guluronic acids , their ionic character is due to carboxyl groups, it is widely used in immobilization of enzymes by the entrapment method" [23,24].

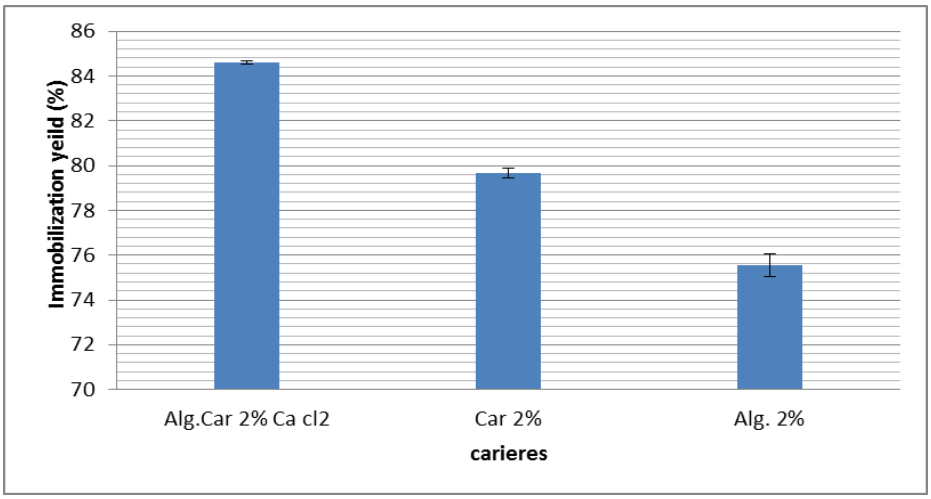

Figure 1:. Immobilization of inulinase on different carriers

3.2 Operational Stability

Fig. 2 shows that the residual activity of the immobilized inulinase on three carriers used for 20 runs was about 88.4%, 75.5 % and 65.8 % when used on alginate/k-Carrageenan 2% and alginate 2% carriers, respectively. The number of runs is higher than that reported for immobilized inulinases from some other microbes. Missau *et al*., [24] reported that "30% of the initial activity of the immobilized inulinase on alginate–chitosan was lost after 8 cycles of assays". Also,20% of the" initial inulinase activity was lost after "7th cycle of hydrolysis" [25].

3.3 Effect of pH on the free and immobilized inulinase

The effect of pH on the free and immobilized inulinase on alginate/k-Carrageenan beads are shown in Fig. 3. The data were normalized to 100% relative activity at pH 5.5 and 6.0 for the free and immobilized enzyme, respectively. This result is comparable with pH optima of the free and immobilized inulinase reported by Gill *et al.,* [26]. The lower pH optima are advantageous in industrial fructose syrup preparations, it prevents

formation of undesired color. It is obvious that the immobilized inulinase is more stable than the free one and gives higher activity than the free inulinase.

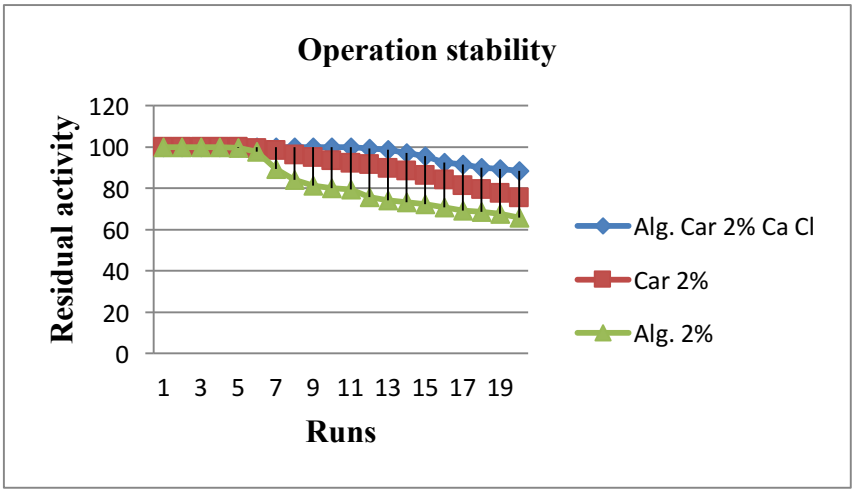

Figure 2: Operation stability of the immobilized inulinase on different carriers.

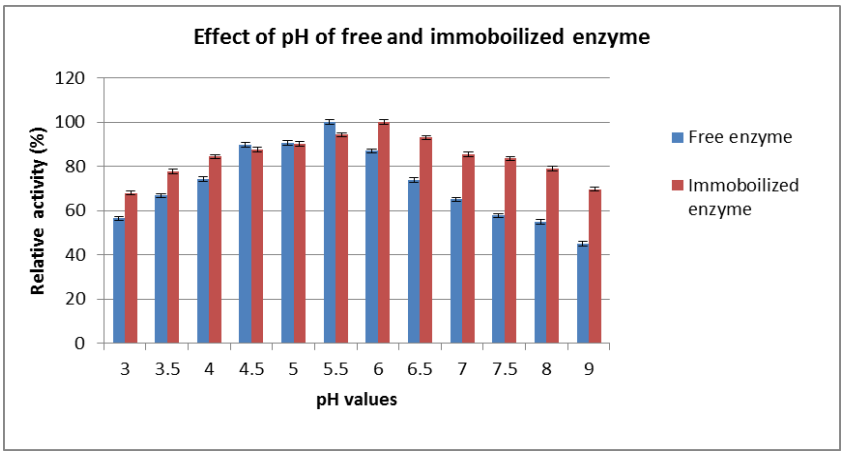

Figure 3: Effect of pH on the free and immobilized inulinase on ALG. CAR beads.

3.4 Effect of different temperatures on the free and immobilized inulinase

The optimal reaction temperature for the immobilized inulinase enzyme shifted from 45°C to 55°C. (Fig. 4). Similar results were reported by Richeti *et al.* [18]. Danial *et al.*, [28] observed that "the optimum temperature for the immobilized inulinase on grafted alginate was at 60 °C". On the other hand Missau *et al.*, [24] achieved "maximum activities for immobilized inulinase on alginate–chitosan beads at 50°C". Also Rocha *et al.*, [27] reported that maximum activities for "immobilized inulinase onto Amberlite IRC 50 was at 50°C".

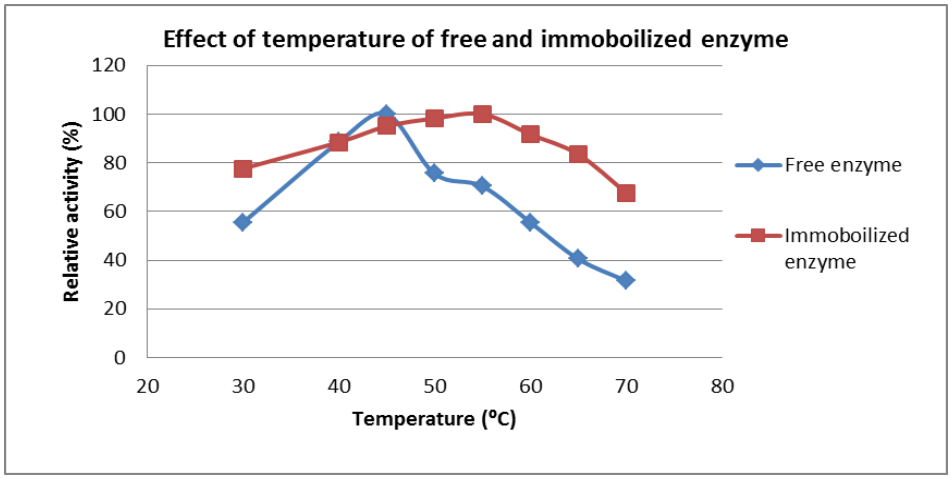

Figure 4: Effect of temperature on the free and immobilize inulinase on Sodium alginate/k-Carrageenan beads.

In this work, the immobilized inulinase retained 67.8% of its activity at 70°C while the free enzyme retained 31.47 % of its activity at the same temperature. The shift of the optimum temperature towards the higher one in the immobilization process, is an indication of the inulinase thermal stability enhancement.

3.5 Thermal stability of the free and immobilized inulinase

The experimental results illustrated in Fig. 5 show that the thermal stability of the immobilized inulinase was improved in comparison to the free form. Thus, the immobilized enzyme retained 76% of its activity at 80°C after 30 minute .While the activity of the free enzyme was reduced to 54% at the same condition. Thermal stability of the immobilized inulinase from *R. oligosporus* NRRL 2549 at 80°C is considerably higher than that reported for immobilized inulinases from some other microbes. "Inulinase produced by *A. niger* remained stable for 30 min at 60 °C" [28]. Nakamura *et al.,* [29] reported that "inulinase extracted from *A. candidus* remained stable for 60 min at 55 °C". The enhanced thermal stability after immobilization is advantageous for the process scale to "lower risk of the bacterial contamination at higher temperature and allows greater solubility of inulin" [21].

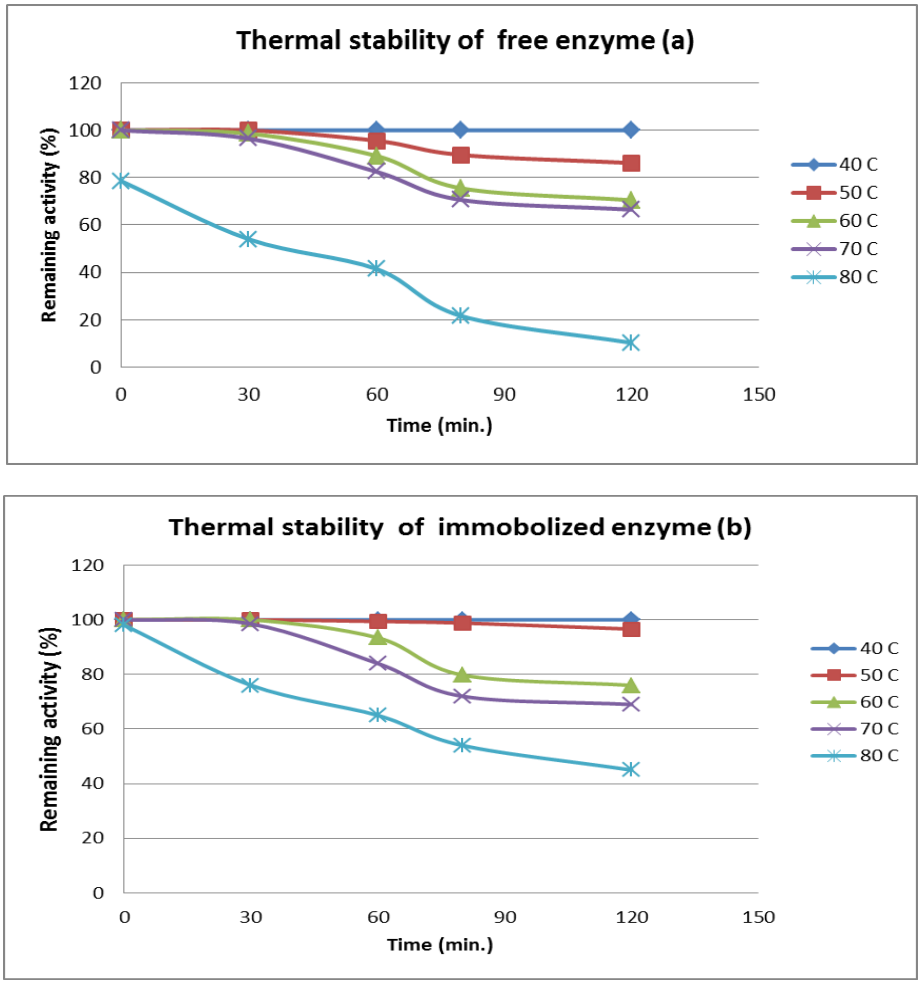

Figure 5: Thermal stability of free inulinase (a) and immobilized inulinase (b) on Sodium alginate/k-Carrageenan beads.

3.6 Continuous inulin hydrolysis by immobilized inulinase

Fig. 6 shows the effect of flow rate [ml/min] and reaction temperature [°C] on percentage of inuline hydrolysis. Continuous inulin hydrolysis is presented in continuous packed bed reactor. Complete inulin hydrolysis was achieved at 50 °C with 0.1 flow rate [ml/min.]. While the inuline hydrolysis reduced to 65% at 30°C with the same flow rate this result similarly to that recorded by [21].

3.7 SEM of Gel beads

SEM results displayed clearly the difference between Alg.car., Alg.car./ PEI, Alg.car./ PEI/ GA and Alg.car./ PEI/ GA/ enzyme. Fig. 7 [A–D] shows the changes which happened in each step. Difference in the surface after each step was noticed clearly. For example in [A] the surface is smooth somewhat and have pores and in [B]

there is accumulation of amine particle on the surface of beads and the pores disappeared , in [C] the GA particles are noticed and in [D] enzyme is immobilized on the surface of Sodium alginate/k-Carrageenan beads.

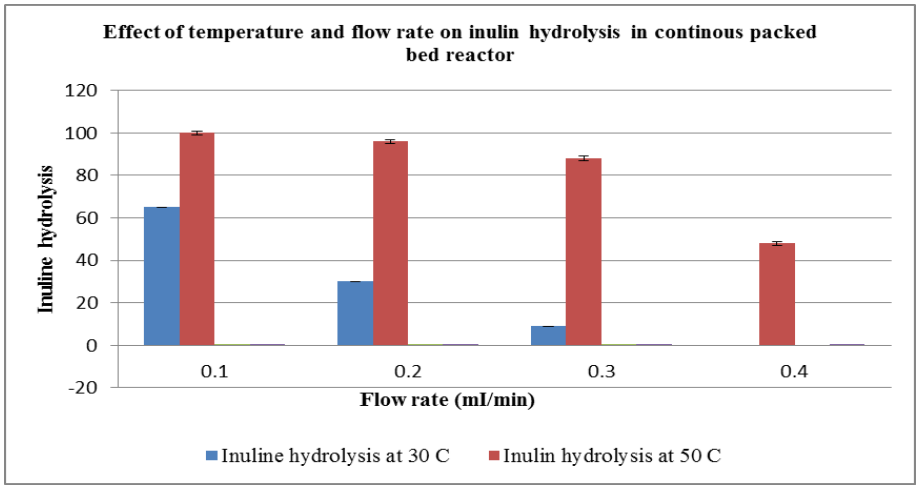

Figure 6: Effect of temperature and flow rate on inulin hydrolysis

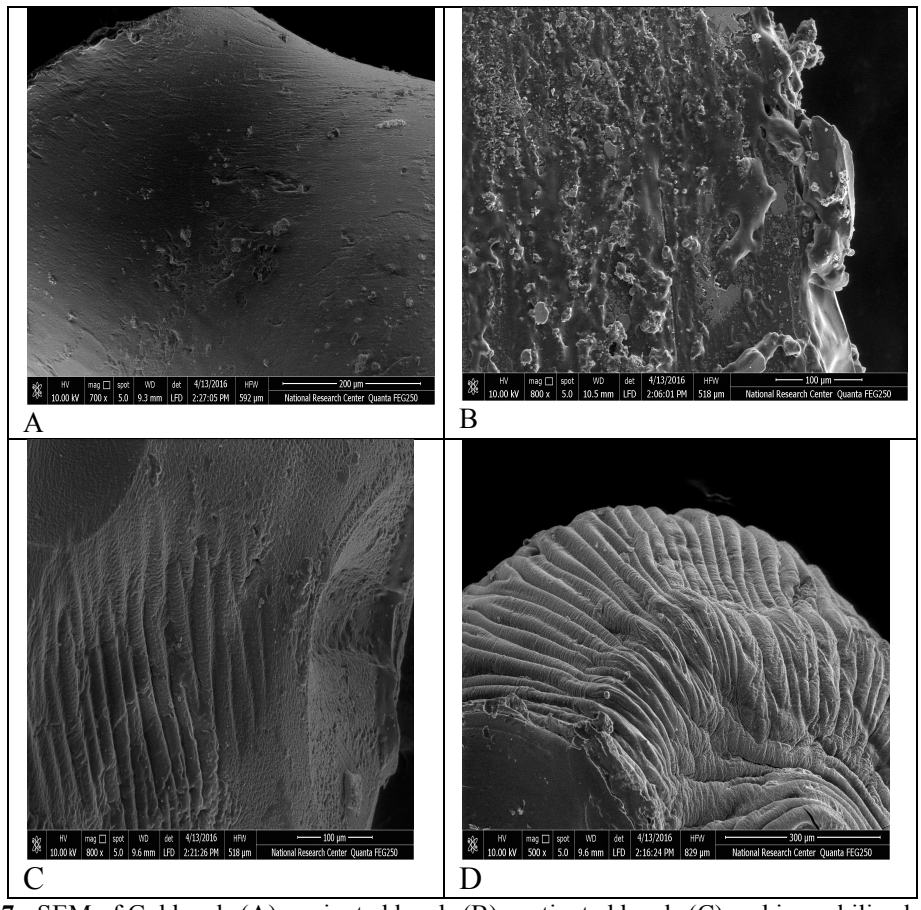

Figure 7: SEM of Gel beads (A), aminated beads (B), activated beads (C) and immobilized one (D).

Conclusion

Crude inulinase enzyme produced by *Rhizopus oligosporus* NRRL 2549 was successfully immobilized on a novel Sodium alginate/k-Carrageenan beads. The optimum pH and temperature of the free and the immobilized inulinase enzyme were studied. Thermal stability showed that the activity of the free enzyme was reduced to 54% at 80˚C after 30 minute while the immobilized form retained 76% of its activity at the same condition. Achieved immobilization yield was 84.6 % and the immobilized enzyme was reused 18 times retaining 90.2% of its activity. Continuous production of fructose from inulin by the immobilized inulinase was investigated in a packed-bed column reactor. Fructose have potential applications in many industrial steps, including food processing, pharmaceutical and medical industries as it consider safe alternative sweetener to sucrose.

References

1. A.J. Cairns, *J. Exp. Bot.,* 54 (2003) 549-567.
2. S. Onodera, N. Shiomi, *Agric. Biol. Chem. J*., 52 (10) (1988) 2569-2576.
3. T. Uchiyama, F.L. Boca Raton, USA: CRC Press-wikipedia, (1993) 169-190.
4. K. Ohta, N. Suetsugu, T. Nakamura, Boca Raton [USA]: CRC Press-wikipedia, (2002) 190-196.
5. Z. Zhang, T. Chi, G. Liu, L. Xue, *Appl. Microbiol. Biotechnol.,* 82 (2009) 211-220.
6. K. Kalra, R. Kumari, *IJSRP.,* 7(3) (2017) 2250-3153.
7. S. Nandagobal, B.D.R. Kumari, *J. American Eurasion Agric.Environ. Sci.,*1 (3) (2006) 225-228.
8. T. Mutanda, B.S. Wilhelmi, C.G. Whiteley, *Enzyme Microb. Technol.,* 43 (2008) 362-368.
9. G.P. Kumar, A. Kunamneni, T. Prabhakar, P. Ellaiah, *World J. Microbiol. Biotechnol*. 21(8) (2005)1359-1361.
10. P.P. Kheng, I.O. Omar, J. Songklanakarin, *Sci. Technol.,* 27(2) (2004) 326- 336.
11. T. Nakamura, K. Kuramori, N. Zaita, H. Akimoto, K. Ohta, *Bull. Fac. Agric. Univ. Miyazaki*. 48 (2001) 49-58.
12. C. Barthomeuf, F. Regerat, H. Pourrat, *J. Ferment. Bioeng.,* 72 (1991) 491- 494.
13. R.A.B. Pessoni, R.L.C. Figueiredo-Ribeiro, M.R. Braga, *J. Appl. Microbiol.* 87, (1999) 14l-147.
14. R.J. Rouwenhorst, L.E. Visser, W.A. Scheffers, J.P. Van Dijken, *Appl. Environ. Microbiol.* 54 (5) (1988) 1131-1137.
15. G. Ongen-Baysal, S.S. Sukan, *Biotechnol. Lett.,* 18 (12) (2008) 1431-1434.
16. B. Kim, H. Kim, S. Nam, *Biotechnol. Bioprocess. Eng.,* 2 (1997) 90-93.
17. Singh P., Gill P.K., *Food Technol. Biotechnol. J.,* 44 [2] (2006) 151-162.
18. A. Richetti, B.C. Munaretto, A.L. Lerin, L.V. Batistella, J. ladimir, D. Oliveira, D.V. Astolfi, M. Luccio, A.M. Mazutti, *Bioprocess Biosyst. Eng.* 35(3) (2012) 383-388.
19. Z. Zhou, G. Li, Y. Li, *Int.J. Biol. Macromol.* 47 (2010) 21-26.
20. G.L. Miller, *Anal. Chem.* 3 (1959) 1426-428.
21. T. Yewale, S.R. Singhal, A.A. Vaidya, *Biocatal. Agric. Biotechnol.,* 2 (2) (2013) 96-101.
22. N. Laowklom, R.C. Pairoh Pinphanichakarn, *Natural Resources.* 3 (2012) 137-144.
23. E.N Danial, M.M.M. Elnashar, G.E.A. Awad, *Ind. Eng. Chem. Res.* 49 (2010) 3120-3125.
24. J. Missau, J.A. Scheid, E.L., L.S. Foletto Jahn, A.M. Mazutti, C.R. Kuhn, *Chem. Process* 2 (2014) 13.
25. R.S. Singh, R. Dhaliwal, M.J. Puri, *Ind. Microbiol. Biotechnol.,* 34 (2007) 649-655.
26. K.p. Gill, K.R. Manhas, P. Singh, *J. Food Eng.* 76 (2006) 369-375.
27. J.R. Rocha, R. Catana, B.S. Ferreira, J.M.S. Cabral, P. Fernandes, *J. Food Chem.,* 95 (2006)77-82.
28. A. Kochhar, A. Gupta, K.N. Kaur, *J. Sci. Food and Agric.,* 79 (1999) 549-554.
29. T. Nakamura, Y. Ogata, A. Shitara, A. Nakamura, K. Ohta, *J. Ferment Bioeng.* 80 (1995) 164-169.