ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2021; 26(3): 2685-2691
doi: 10.25083/rbl/26.3/2685.2691

Received for publication, December, 8, 20209
Accepted, January, 20, 2021

Original paper

Production, purification and immobilization of inulinases from Aspergillius species

MIHAELA CARMEN EREMIA[1*] and MARIA-MONICA PETRESCU[1]

[1]Department of Pharmaceutical Biotechnology, National Institute for Chemical-Pharmaceutical Research and Development-ICCF, Bucharest, Romania

Abstract Inulinases are enzymes catalysing hydrolysis of polyfructosans to produce fructose or fructooligosaccharides; these properties have attracted interest of many researchers towards exploring various plant sources (agro food waste) as substrates. According to the literature, various microorganisms, such as fungi, yeast, bacteria, and actinomycetes, can synthesize inulinase. Producer microorganisms can be the micromycetes Aspergillus, Penicillium, Rhizopus and Fusarium, the yeast Kluyveromyces and the bacteria *Clostridium thermosuccinogenes* and *Bacillus subtilis*, using inulin, sucrose, fructose, lactose, raffinose, xylose as sources of carbon. The present study mainly envisages inulinase produced by Aspergillius species grown on a fermentation medium based on different carbon sources (inulin and agro-alimentary by-products). The crude extract was purified by fractional precipitation with ammonium sulphate. The dissolved and dialyzed precipitate was further purified by ion exchange chromatography on DEAE-Sephadex. The experiments led to the obtaining of an inulinase with a specific activity of 43.34 U/mg proteins. Inulinase immobilization tests on chitosan beads have led to a stable biocatalyst at temperature and pH variations that could be useful for obtaining fructose syrup.

Keywords Inulinase, *Aspergillius species*, biosynthesis, immobilization, inulin.

To cite this article: CARMEN EREMIA M, PETRESCU MM. Production, purification and immobilization of inulinases from *Aspergillius species*. *Rom Biotechnol Lett*. 2021; 26(3): 2685-2691. DOI: 10.25083/rbl/26.3/2685-2691

✉ *Corresponding author: MIHAELA CARMEN EREMIA, National Institute for Chemical-Pharmaceutical Research and Development-ICCF, Bucharest, 112, Vitan Av., RO-031299, District 3, Bucharest, Romania
E-mail: mihaelaceremia@yahoo.com

Introduction

In recent years, researchers have turned their efforts to producing natural polysaccharides due to an increase in metabolic disorders (diabetes, obesity, cardiovascular disease, hyperlipoproteinemia, hypocalcemia, gout). Inulinases are important hydrolysing enzymes, which specifically act on β-2, 1 linkages of inulin to produce fructose or fructooligosaccharides. Inulinases can be produced by a lot of microorganisms, including bacteria, fungi and yeast (NIROBOL LAOWKLOM & al [1], YUN J.W. & al [2], GAVRAILOV & IVANOVA [3], RAM SARUP SINGH and KANIKA CHAUHAN [4]). High optimum temperature (60°C) and thermostability are two important criteria that determine suitability of this enzyme for industrial applications, which are also influenced by the enzyme cost, a major limiting factor.

Among various carbon sources employed for enzyme production, inulin-containing plant materials offer advantages in comparison to pure substrates. Inulin is a polysaccharide of vegetable, used as a substrate in the food industry, for the production of fructose-rich syrups. (NIROBOL LAOWKLOM & al [1], YUN J.W. & al [2], RAM SARUP SINGH and KANIKA CHAUHAN [4]; GAVRAILOV & IVANOVA [3], GERN, R.M.M. & al [5]), usable in the dietary sector. Also, the various oligosaccharides derived from inulin find their application in the medical sector. The pharmaceutical importance of short-chain fructooligosaccharides and inulooligosaccharides leads to the expansion of their industrial production (MARIA ROSA VELA SEBASTIÃO FERNANDES and BO JIANG [6], GUIMARAES L.H.S. & al [7]). These compounds, known as prebiotics, have functional properties and hence health benefits if consumed in recommended dosages. These prebiotics can be produced enzymatically by sucrose elongation or via enzymatic hydrolysis of inulin by exoinulinases and endoinulinases, acting alone or synergistically. Additionally, there are many reports on the production of various metabolites like citric acid, lactic acid, ethanol, biofuels, butanediol etc. using mixed cultures of inulinase producing organisms with other microorganisms (CHI Z.M. & al [8]; ZHENMING CHI & al [9]).

The present study mainly refers to inulinase production by *Aspergillius* species, including bioprocess optimization, purification, characterization, enzyme immobilisation and its application in oligosaccharides production from agricultural waste. In order to improve enzyme biosynthesis we have tried different substrates and optimization methods (nitrogen sources, temperature, pH, bioprocess duration).

Recovery and recycling of enzyme are very important for the industrial application of the biocatalyst. In the literature are studied different immobilization techniques, as well as the properties of inulinase immobilized on different supports and compared with enzyme in native state (TATYASO YEWALE & al [10]; MARCELI FERNANDES SILVA & al [11]).

Materials and Methods

1. Biological Material

Eleven strains of filamentous fungi (*Aspergillus awamory ICCF 259, Aspergillus flavus ICCF 233, Aspergillus oryzae ICCF 198, Aspergillus niger ICCF 92, Aspergillus nigricans ICCF 402, Aspergillus phoenicis ICCF 207, Aspergillus terreus ICCF 262, Aspergillus sydowi ICCF 236, Rhizopus stolonifer ICCF 223, Trichoderma harzianum ICCF 179, Trichoderma viride ICCF 177)* from the Culture Collection of Industrial Importance Microorganisms of the National Institute for Chemical & Pharmaceutical Research and Development (PETRESCU MARIA and EREMIA MIHAELA-CARMEN [12]) were investigated. The strains were maintained in agar slant tubes and in Petri dishes at 4°C in their respective maintenance media: PDA (Potato Dextrose Agar).

2. Cultivation

Each one of the isolates positive for inulinase activity was further transferred from the pre-inoculum slant tubes to 100 ml of YMPG in 500 ml Erlenmeyer flask and cultivated aerobically for 24 hours up to 72 hours at 30°C, for the inoculum production. Each inoculum was used at a final concentration of 2% to inoculate 500 ml Erlenmeyer flasks containing 100 ml of the fermentative medium consisting of M2 or M3 medium supplemented with 2% (g/v) inulin or banana peels and orange peels, as inductive substrates. The fermentation broth contained: yeast extract 2% (M2) or corn steep liquor 2% (M3), NH_4NO_3 0,3%, $(NH_4)_2HPO_4$ 0,4%, KH_2PO_4 0,1% and $MgSO_4$ 0,05%. The cultures were grown by shaking at 220 rpm, 30°C for seven days. Then, the mycelia were separated by centrifugation at 4°C and 8000 rpm for 20 minutes and then the supernatant was assayed for enzyme activity and used for enzyme isolation and purification.

3. Inulinase screening

The capacity of the 11 strains of fungi to metabolise inulin as a carbon source was evaluated by using Durham test tubes. Small test tubes are placed in an inverted position within the ordinary test tubes used for cultivations. The larger tube is then filled with medium, plugged in and sterilised. The medium used contained 0.5% yeast extract and 1% inulin. The evaluation of the degraded inulin was observed as accumulation of gas in the small inverted tube.

4. Enzyme Purification

The first stage of post-biosynthesis processing consist in fractionated precipitation with ammonium sulphate in two steps: 60% and 80% saturated ammonium sulphate. After the first step, the formed precipitate without enzymatic activity was discarded. The precipitate obtained at 80% ammonium sulphate saturation was speared by centrifugation and dissolved in a small volume of deionized water; the resulting solution was dialyzed for 24 h against

distilled water and for another 24 h against 50 mM sodium acetate buffer (pH 4.7). This fraction was submitted to chromatography in a DEAE-Sephadex A-50 column, previously equilibrated with 50 mM acetate buffer, pH 4.7. The proteins were eluted with stepwise gradient concentrations of NaCl range from 0.1 to 0.5 M prepared in the same buffer at a flow rate of 1 ml/1 min and collected in 5 ml fractions. The fractions were analysed and those containing enzyme activity were collected and lyophilized (RAM SARUP SINGH and KANIKA CHAUHAN [4]; PESSONI, A.B. & al [13]).

5. Enzyme Immobilization

Chitosan beads were prepared as follows: 1 g of chitosan was dissolved in 50 ml of 0.1 M acetate buffer, pH 4.0. Different concentrations of chitosan gel: 1, 1.5 and 2%, respectively, were tested. The solution was extruded dropwise through a hypodermic needle using a peristaltic pump into a glass containing a mixture of distilled water, methanol and 0.1 N NaOH solution (4 : 5 : 1) to obtain chitosan beads. The beads were allowed to mature overnight at 30°C, then washed with distilled water and stored in 50 mM acetate buffer, pH 4.7, at 4°C, till further use.

Immobilization of inulinase on chitosan was performed in two steps. The inulinase stored in lyophilized form was solubilized in acetate buffer (pH 4.7), and left for 1 h, with 120 rpm stirring. The complex enzyme-support was centrifuged, and the beads were washed with a 0.5 M saline solution (TATYASO YEWALE & al [10]).

In the second step, the adsorbed enzyme was covalently fixed on the support by adding a solution of glutaraldehyde prepared in 50 mM acetate buffer (pH 4.7) until a final concentration of 1.66% is reached. This mixture was incubated for 1 h at 30°C with gentle stirring and then it was centrifuged. This immobilized enzyme was washed with 0.5 M saline and stored at 4°C till further use.

6. Analytical Methods

The inulinase activity was tested according to a method described by Miller (MILLER, G.L. & al [14]), as follows: enzyme solution (0.1 ml) was mixed with 2% inulin (0.9 ml) in 0.1 M acetate buffer, pH 5.5. The reaction takes place at 50°C for 15 min.

For the immobilized enzyme the analysis of the inulinase activity is done as follows: immobilized enzyme (0.5 g) were added in 2% inulin (4.5 ml) in 0.1 M acetate buffer, pH 5.5, and incubated at 50°C, with 150 rpm stirring, for 30 min.

The reducing sugars were determined by the dinitrosalicylic acid method. For this, 2 ml of DNSA reagent was added to each tube were placed in boiling water for 5 min to stop the enzyme activity. Each sample was cooled to room temperature. Absorbance was determined in a spectrophotometer at 540 nm. The definition of a unit of activity is: one activity unit is defined as the amount of enzyme required to produce one micromole of reducing sugar per minute, under assay conditions (PETRESCU MARIA and EREMIA MIHAELA-CARMEN [12]).

The protein content was determined by Lowry method using BSA as a standard (LOWRY, O.H. & al [15]).

Immobilization efficiency was defined as:

$$\frac{\text{specific activity bound on chitosan}}{\text{specific activity initially added}} \times 100$$

The partial characterization of immobilization inulinase of *A. terreus* was performed in terms of thermal and pH stability.

The thermal stability of immobilized inulinase was evaluated by incubation of the enzyme in acetate buffer (0.1 M, pH 5.5) at 40, 50, 60 and 70°C. Samples were analysed after 15 min for inulinase activity measurement.

The stability of the immobilized inulinase at different pH values was tested by incubation in sodium acetate buffer with pH values of 4.0, 4.5, 5.0 and 5.5 at 50°C. Samples were analysed for inulinase activity was determined as described previously.

Results and Discussion

1. Cell growth and inulinase microbial biosynthesis on conventional media

The fungal strains *Aspergillius sp.* was highlighted, presenting the largest volume of gas produced by the metabolism of inulin from the environment in the experiment Durham (DURHAM HE [16]). As seen in Figure 1, *Aspergillus awamori*, *Aspergillus niger*, *Aspergillus nigricans* and *Aspergillus terreus* strains had a higher activity than the other four tested, as well as the best cell growth, indicating that these strains can metabolize inulin as the only carbon source.

In order to optimize the conditions for inulinase biosynthesis with the selected strains, researches were carried out on the type of inoculum (Ig - inoculum with the sole carbon source of glucose and Ii - inoculum with the only source of carbon inulin) and the preferred nitrogen source (organic or inorganic). The results are shown in Figure 2.

The *Asp awamori* strain had the highest growth in terms of biomass on the M2 medium, regardless of the type of inoculum. However, the enzymatic activity was higher on the M3 medium; it seems that the presence of the organic N source (corn steep liquor) was beneficial to the enzyme. Also, an increase in activity can be observed when using inulin as an inducer in the inoculum phase.

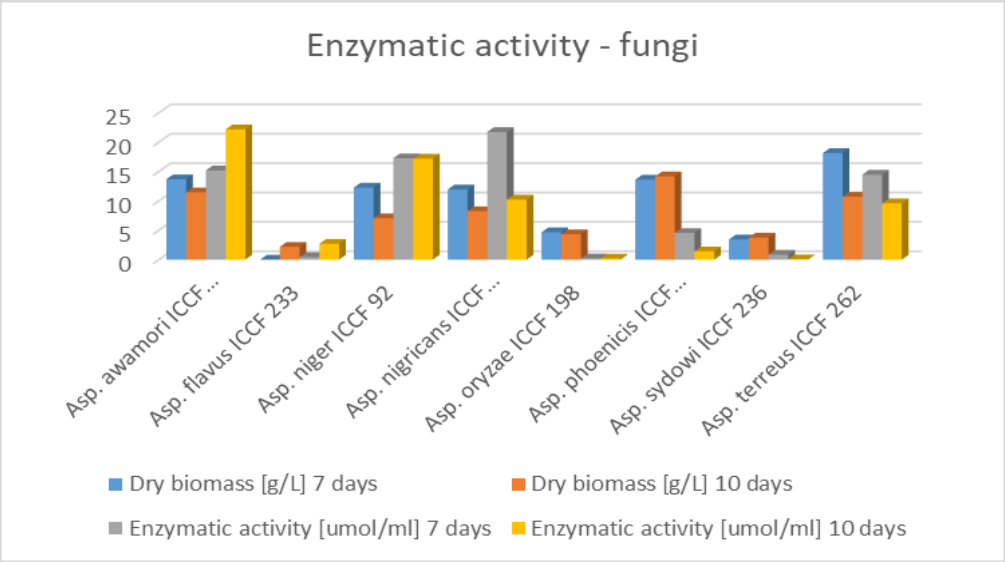

Figure 1. Biomass development and fungus enzymatic activity.

The biomass production of *A. niger* strain was significantly improved by using the Ig-M2 variant, but enzymatic activity was higher on the Ig-M3 variant. For this strain, it can be concluded that the use of inulin since the inoculation phase does not positively influence the development of biomass or enzymatic activity.

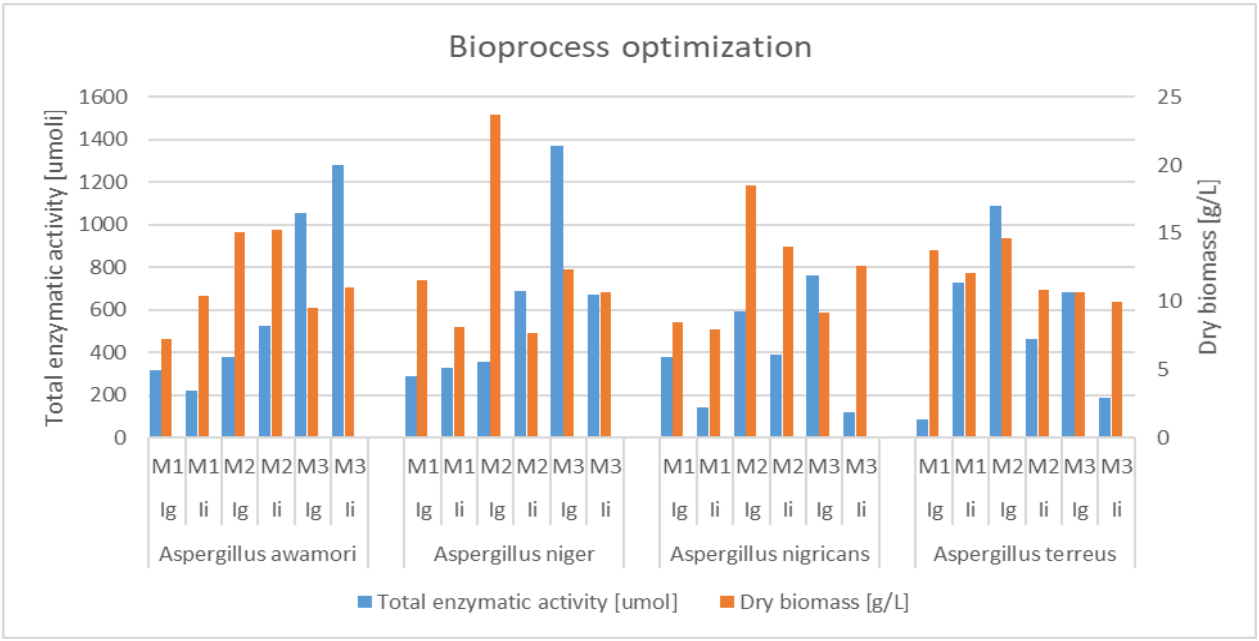

Figure 2. Bioprocess optimization

The *A. nigricans* strain developed best M2 medium variant and as in case of *A. niger*, the enzymatic activity was higher on the Ig-M3 variant.

The *A. terreus* strain had a relatively uniform distribution in terms of biomass development on the selected medium variants and enzymatic activity was higher on the Ig-M2 variant. It is the only strain where we found a correlation between biomass development and enzymatic activity on the same culture medium.

2. Influence of other C source on cell growth and enzyme production

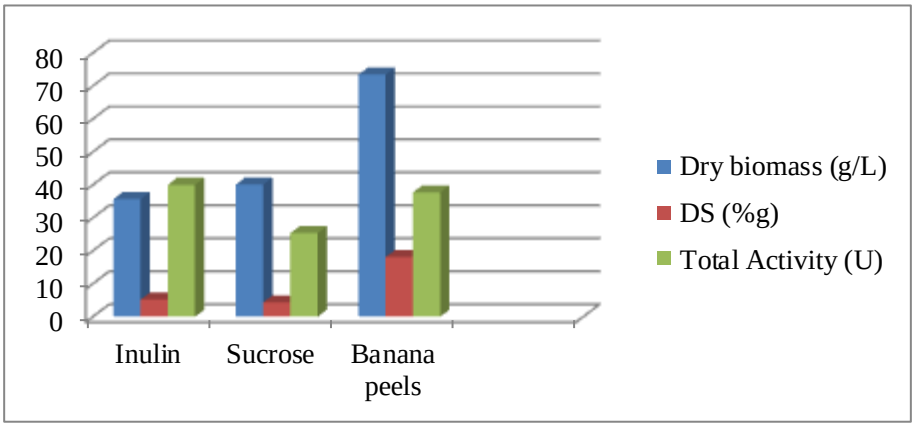

Figure 3. The influence of C source on inulinase production by *Aspergillius awamori*.

The strain developed best in terms of biomass on the medium variant with banana peels, however, the enzymatic activity had similar values as in the medium that used inulin as carbon source.

The strain has significantly improved enzymatic activity on the medium variant that contained orange peels as its source of carbon. Regarding the comparison of the two *Aspergillius* strains, one could observe the much higher activity of fungi (EA close to 300 U and EA around 40 U for *A. awamori*). For this reason, the study will continue with the strain *A. terreus*.

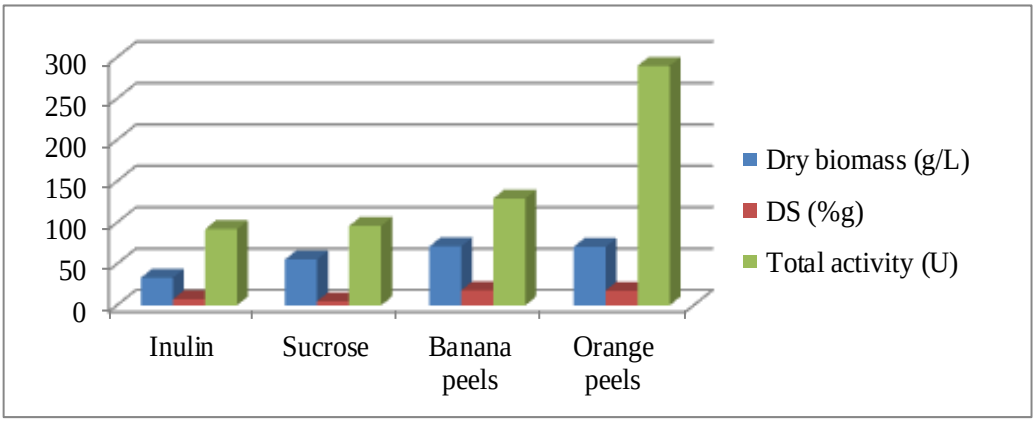

Figure 4. The influence of C source on inulinase production by *Aspergillius awamori*.

3. Inulinase purification

The supernatant obtained by centrifugation of *A. terreus* culture, showing inulinase activity, was purified by two successive steps: fractionated precipitation with ammonium sulphate and ion exchange chromatography on DEAE-Sephadex (Table 1).

By adding ammonium sulphate to the enzyme containing supernatant as to obtain a final salt saturation of 60%, a first stage of purification occurs, because the obtained precipitate has not enzymatic activity, whereas inulinase is found in the liquid phase. Supplementing the ammonium sulphate content of this liquid phase till 80% saturation, the enzyme precipitates, thus being separated from other impurities. This final precipitate shows a relative high inulinase activity (about 27.6% of initial activity).

Table 1. Purification of the inulinase from *Aspergillius terreus*

Stages	Volume (ml)	Activity (U/ml)	Total activity (U)	Protein (mg/ml)	Specific activity (U/mg)	Purification factor
Crude enzyme	570	5.12	2918	1.14	3.66	1.00
Precipitation with $(NH_4)_2SO_4$	30	27.5	825	1.04	26.44	7.22
Dialysis	34	23.7	805.8	0.98	24.18	6.61
Purification with DEAE-Sephadex	17	15.6	567	0.36	43.34	11.84

In the second stage, the enzyme obtained after the precipitation with $(NH_4)_2SO_4$ and dialyzed, was loaded into a DEAE-Sephadex column, on which inulinase eluted was purified over 11.84-fold, with yield 70.4%. Finally, the purified enzyme was lyophilized.

4. Inulinase immobilization

To attach the inulinase to chitosan, we perform firstly an adsorption step, at a slight acid pH, achieving an enzyme loading of 83 U/g support. Then, the experiments aiming to covalently fix the enzyme in chitosan, using different concentration of glutaraldehyde, pointed out that the best conditions for an efficient binding are: 1% glutaraldehyde and 1 hour incubation time (Table 2).

5. Partial characterization of immobilization inulinase

As a result of thermal stability determination experiments, it was observed there are not significant losses of activity.

Table 2. Immobilization of inulinase on chitosan beads

Experiment	Initial activity (U)	Immobilized activity (U/g support)	Yield (%)
Immobilization on chitosan gel 1%, crosslinking with GA	317	263.11	83
Immobilization on chitosan gel 1.5%, crosslinking with GA	317	244.09	77
Immobilization on chitosan gel 2%, crosslinking with GA	317	209.22	66

The influence of pH on the stability of immobilized inulinase was verified in the range 4.0 to 5.5. The results obtained indicated a 25-30% loss of activity between pH 4-4.5, which confirm the pH = 5 as the optimum value for inulinase activity. The analysis of the results obtained was presented higher stability at slightly acidic pH with a loss of activity (70-76%).

Our studies on stability of immobilized inulinase found an optimal the temperature at 50°C and pH value of 5.0 for enzymatic reactions efficiency.

Conclusion

From eight strains *of Aspergillus sp.* tested as inulinase producers, *Aspergillus awamori* and *Aspergillius terreus* strains were selected as the most important, according to the study of biomass development and inulinase production.

Study of the bioprocess optimization for obtaining inulinase with selected strains carried out on enzymatic activities has been performed out with different sources carbon, namely: inulin, sucrose and agro-alimentary by-products (banana and orange peel). These experiments pointed out that *A. terreus* strain produces inulinase with higher activity than the enzyme produsd by *A. awamori* on all media studied.

As a result of post-biosynthesis processing of the fermentation medium, by successive three-step purification, a solution with an inulinulase content of 92.2 U/ml with a yield of 30% was obtained.

Inulinase immobilization on chitosan by adsorption combined with glutaraldehide cross-linking, led to a preparation characterized by 82.87 U/g support, with immobilization yields between 77 and 87%.

Acknowledgements

This paper is financially supported by the National Institute for Chemical-Pharmaceutical Research and Development through the dissemination activity of research results.

Conflict of Interest

The authors have no conflict of interest to declare.

References

1. NIROBOL LAOWKLOM, RUNGTRAKARN CHANTANAPHAN, PAIROH PINPHANICHAKARN. Production, Purification and Characterization of Inulinase from a Newly Isolated Streptomyces sp. CP01, *Natural Resources*, 3, pp. 137-14 (2012).
2. YUN, J.W., D.H. KIM, T.B. UHM and S.K. SONG. Production of high-content inulo-oligosaccharides from inulin by a purified endoinulinase. *Biotechnol. Lett.*, 19: 935-938 (1997).
3. GAVRAILOV & IVANOVA. Biosynthesis of inulinases by *Bacillus* bacteria, *J. BioSci. Biotech.* SE/ONLINE: 75-82 (2014).
4. RAM SARUP SINGH and KANIKA CHAUHAN. Production, Purification, Characterization and Application of Fungal Inulinases, *Current Biotechnology*, 5, pp. 1-19 (2016).
5. GERN, R.M.M., FURLAN, S.A., NINOW, J.L., and JONAS, R., sppl. *Microbiol. Biotechnol*., vol. 55, no. 5, pp. 632-635 (2001).
6. MARIA ROSA VELA SEBASTIÃO FERNANDES and BO JIANG. Fungal Inulinases as Potential Enzymes for Application in the Food Industry, *Advance Journal of Food Science and Technology* 5(8): 1031-104 (2013).
7. GUIMARAES L.H.S., TERENZI H.F., POLIZELI M.L., JORGE J.A. Production and characterization of a thermostable extracellular β-Dfructofuranosidase produced by *Aspergillus ochraceus* with agroindustrial residues as carbon sources, *Enzyme and Microbial Technology*, 42, 52-57 (2007).
8. CHI Z.M., ZHANG T., CAO T.S., LIU X.Y., CUI W., ZHAO C.H. Biotechnological potential of inulin for bioprocesses, *Bioresource Technology*, 102, 4295-4303 (2011).
9. ZHENMING CHI & ZHE CHI & TONG ZHANG & GUANGLEI LIU & LIXI YUE. Inulinase-expressing microorganisms and applications of inulinases, *Appl Microbiol Biotechnol*, 82: 211220, (2009).
10. TATYASO YEWALE, REKHA S. SINGHAL, ALANKAR A. VAIDYA. Immobilization of inulinase from *Aspergillus niger* NCIM 945 on chitosan and its application in continuous inulin hydrolysis, *Biocatalysis and agricultural Biotechnology*, vol. 2, pp. 96-101 (2013).
11. MARCELI FERNANDES SILVA, DIANE RIGO, VINÍCIUS MOSSI, ROGÉRIO MARCOS DALLAGO, PEDRO HENRICK, GRACIELE DE OLIVEIRA KUHN, CLARISSA DALLA ROSA, DÉBORA OLIVEIRA, J. VLADIMIR OLIVEIRA, HELEN TREICHEL. Evaluation of enzymatic activity of commercial inulinase from Aspergillus niger immobilized in polyurethane foam, food and bioproducts processing, (91), 54-59 (2013).
12. MARIA PETRESCU, MIHAELA-CARMEN EREMIA. Effects of gamma radiation on inulinase production by *Aspergillus terreus*, Scientific Bulletin. *Series F. Biotechnologies*, Vol. XXII, 2018, ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364.
13. PESSONI, A.B., BRAGA M.R. and FIGUEIREDO-RIBEIRO R.C.L. Purification and exo-inulinases from *Penicillium janczewskii* growing on distinct carbon sources. *Mycological*, 99: 493-503 (2007).
14. MILLER, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem.*, 31: 426-428 (1959).
15. LOWRY, O.H., FARR, A.L., RANDALL, R.J. Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, 193, 265-275 (1951).
16. DURHAM H.E. A simple method for demonstrating the production of gas by bacteria, *British Medical Journal*. 1898, 1:138 (1952).