# Substrate Optimiztion For High Yield Of Inulinase By Submerged and Soild State Fermentation

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# Abstract

# Inulinase is an industrially significant enzyme widely used in the production of fructose syrups from inulin-rich substrates. To enhance the yield of inulinase, this study focused on substrate optimization using two fermentation techniques: submerged fermentation (SmF) and solid-state fermentation (SSF). The aim was to compare and optimize the conditions for inulinase production using different substrates and fermentation approaches. For submerged fermentation, various carbon and nitrogen sources were tested, and the effects of pH, temperature, and incubation time were investigated. Additionally, solid-state fermentation was explored using different solid substrates and moisture levels to determine their impact on inulinase production. Results showed that both SmF and SSF demonstrated the potential for inulinase production, but their optimal conditions differed significantly. In SmF, the highest inulinase yield was achieved with a specific combination of carbon and nitrogen sources at a specific pH and temperature. Conversely, SSF yielded maximum inulinase production with certain solid substrates and precise moisture content. This study offers valuable insights into substrate optimization for inulinase production through SmF and SSF approaches, providing practical guidelines for industrial applications. The findings highlight the significance of tailoring fermentation techniques to specific substrates, enabling high yields of inulinase for efficient fructose syrup production.

# Keywords:

# Inulinase, substrate optimization, submerged fermentation, solid-state fermentation, fructose syrups, carbon sources, nitrogen sources, pH, temperature, moisture content, industrial enzymes.

# INTRODUCTION

Inulin is a type of fermentable fiber that is found naturally in the roots of many foods, such as whole wheat, onions, garlic, and artichokes, and is commonly extracted from chicory root and added to foods. Dietary fibers can promote [gut health](https://www.verywellhealth.com/how-to-have-healthy-gut-bacteria-1945326), increase feelings of fullness, aid in weight loss, and improve [heart health](https://www.verywellhealth.com/how-to-keep-your-heart-happy-1746310) by [reducing cholesterol](https://www.verywellhealth.com/six-ways-you-can-lower-high-cholesterol-697612). Inulin is a type of oligosaccharide called a fructan. Fructans are a chain of fructose (sugar) molecules strung together. Inulin is considered a functional food, and adding it to your diet may improve your health. Inulin is classified as a prebiotic because of its ability to stimulate the growth of beneficial bacteria such as Bifidobacteria. inulin fibers may protect or delay type 1 diabetes in mice by modulating the immune response and improving gut health (Hume 2017). Inulin, a type of fiber, may also help to control appetite by increasing feelings of fullness. Inulin, a fructan-type polysaccharide, consists of (2→1) linked -d-fructosyl residues (n = 2–60), usuallywith and-glucose end group. The molecule almost exclusively linear, with only a few percent branching (Kays, Stanley 2007). Because of the β(2,1) linkages, inulin is not digested by enzymes in the human alimentary system, contributing to its functional properties: reduced calorie value, dietary fiber, and prebiotic effects. The enzymatic digestion of inulin (fructans) yields fructose syrups, which have been reported to have beneficial health applications in humans. Research on root associated bacteria has been carried on several types of plants for several years. Several strains of rhizoplane bacteria could increase growth and protect plants from pests and diseases. The role of rhizoplane bacteria from the onion plant still need further exploration on enzyme production.

**MATERIALS AND METHOD**

**Isolation of *A. cepa*-associated bacteria** (Lai et al., 2008).

Onion plants (A. cepaLinn., cultivar) were collected from a farmer’s plantation in valkai, Thiruvarur district. To isolate onion root endophytes, the roots were rinsed with distilled water to remove debris and then sterilized with 75% alcohol for 15 min, followed by immersion in 6% sodium hypochlorite for another 15 min. Roots were washed with sterilized water after the sterilization process. Samples were ground as described previously The extract was serially diluted 10-fold over a range between 10–2 and 10–8, and a 100-μL cell suspension was plated onto nutrient agar (HiMedia) plates

## Isolation of Strain

Microorganism from onion root surface adhered soil(rhizoplane) was collected and serially diluted. 10& was plated on modified nutrient agar plate and medium composition was comprised off the following: Yeast extract 3.0 g; peptone Nacl 5 g; 10.0 g; Glucose 4.0 g; Agar 20.0 g; Distilled water 1.0 L; pH 7.2–7.4. after incubation colony morphology were recorded and screened for inulinase production

## Screening of inulinases activity

The selected bacterial species were inoculated separately into the modified Yeast glucose agar with inulin (0.5%). Plates were incubated at 37 °C for 24 h days. The cultures flooded with Lugos iodine and incubated for 5min.after washing with water further incubated 1 h and analyzed for activities of inulinases

## Pretreatment of Substrate

1. Garlic (bulbs) were washed thoroughly with cold water, sliced, and then dried under shadow. After drying further dried at 70°C for 2 h. The dried slices were then milled to a fine powder with a hammer mill. After milling, the resultant powder was used directly as a carbon source
2. CORN cob was collected and dried well and grined into fine poeder. The substrate is stoered in sterile container

## Substrate fermentation

Fermentation was carried out in Erlenmeyer flasks (250 mL) with 2 g of pretreated garlic powder, supplemented with 1g of peptone, 1g Yeast extract, 0.1 g K2HPO4 nutrients defined by the experimental design. Moisture was adjusted to 50%. Each flask was covered with hydrophobic cotton and autoclaved at 121°C for 20 min. After cooling, each flask was inoculated with 2 mL of 24 h old the bacterial suspension previously prepared and incubated for 48 hrs. similar set up was maintained for corn cob and pure inulin alone.

## Submerged Fermentation

Submerged fermentation of was carried out on a rotator shaker at 150 rpm at 37°C in 250ml of Erlenmeyer flask that filled nutrient broth 100 mL medium with 1% substrate. The ferementation devided into 6 groups as bellow. The medium is autoclaved at 121°C for 20 minutes. All the flask was inoculated by Bacillus sp. The samples were collected after 48 h. The samples were centrifuge at 5000 rpm for 15 minutes. The supernatant was used as enzyme extract and used for enzyme assay.

|  |  |
| --- | --- |
| SMF 1 | Inulin |
| SMF2 | Corn cob |
| SMF 3 | garlic |
| SMF 4 | Corn cob+inulin |
| SMF5 | garlic+inulin |
| SMF6 | garlic+cob |

## Extraction of Inulinase

After fermentation, 5 volumes of distilled water were added to the fermented matter, and the contents were agitated for 30 minutes at 200 rpm on a rotary shaker (at 28°C). Then the sample was centrifuged at 15000 rpm for 20 minutes, and the supernatants were used for enzyme assay

## Assay of Enzyme Activity

Enzymes were assayed by measuring the concentration of reducing sugars released from inulin or sucrose. Both culture filtrate and protein isolate were used. The reaction mixture containing 1 mL of diluted crude enzyme and 4 mL of 2% inulin or 2% sucrose (dissolved in 0.1 M acetate buffer, pH 5.0) was incubated at 50°C. After incubating for 30 min, aliquots of 0.5 mL were withdrawn and increase in reducing sugar was estimated by a 3,5-dinitrosalicylic acid method using calibration curve obtained with a standard solution of fructose. Absorbance was read at 575 nm. A higher absorbance indicated a high level of reducing sugar produced and consequently a high enzyme activity. One unit of inulinase activity

(U) was defined as the amount of enzyme, which forms 1 *μ*mol fructose per min. Results of the determination of inulinase activity were presented in units of activity/gram of dry substrate (U/gds.).

## Biochemical characterization of active strain

Selected keratinolytic bacteria were then characterized by gram staining based on cultural, morphological, as well as biochemical characteristics. For the activities of oxidase, catalase, citrate utilization, indole production, methyl-red (MR), Voges-Proskauer (VP) were performed.

**RESULTS AND DISCUSSION**

Bacterial colonies on Agar plates showed 26 X107 CFU (colony forming unit)/g of soil. Mostly (22 colonies) of the colonies were formed after 24 h and four were developed after 48 h. based on colony morphology (table 1) clolnies were designated as onionsoil bacteria 1-5 (OS1-5). Totally five different colonies were selected (plate 1) and selected for inulinase screening . Out of 5 only OS3 and 5 were KOH mount negative denotes it comes under Gram positive cell wall and also catalase negative. All the isolates were oxidase positive (table 2). Among the all tested bacterial strain (OS1-OS5) OS4 only showed Inulinase positive (plate 2). The biochemical character of OS 4 revals Gram negative, green pigment producing positive on oxidase methyl, and citrate and negative on VP and indole relative to the chractristics of *Pseudomonas fluorescence*. During the primary screening, among the five bacterial isolates from marine water higher hydrolytic zone formation was recorded and enzyme production further optimized on substrate(plate 3) .

The concentration of total protein of cell extract of substrate and submerged fermentation given in table 3. substrate fermented medium gave 16, 28 and mg/L respectively among Inulin ≥ Garlic ≥Corn cob (Fig 1). Similarly concentration of total protein of cell extract of submerged fermented medium shows 14, 22,36,98,112,98 mg/L corresponding to inulin, cob, garlic, cob+inulin, garlic+inunlin and garlic+cob . maximum extracellular protein content was found in submerged fermentation (118 mg/L ;Fig 2) and minimum 14 mg/L (inulin alone). The main counterpart for SSF is the submerged fermentation (Smf), a process in which microorganisms grow in liquid medium, with high content of free water(Farinas, 2015).

In the present study, inulinase enzyme was screened in supernatant as extracellular from substrate fermentation and recorded as 88≥220≥96 U(figure 3). Among the different fermentation the highest activity level of inulinase from *P.fluorescence* was recorded in the supernatant of extracellular enzyme from garlic substrate fermentation . The maximum enzyme activity of Submerged fermentation (Fig 4) was 692 U followed by 660 and 580 respectively for garlic+cob; garlic+inulin and cob+inulin. Substrate of garlic and cobalone gave moderate activity 248 and 122 U nits and less significant in inulin alone (98U).

Our results indicated that, the highest activity level of inulinase was recorded in the supernatant of extracellular submerged fermentation (580-692U/ml) with specific activity of 98 mg/L (cob+in) and 96 mg/L (garlic+cob) protein. But the activity 660 recorded total protein 112 mg/L. The data reveals concentration of total protein does not influence the enzyme activity.

In order to determine the effect of submerged and substrate feremntatoion on enzyme production we used two different source and six different stratagies for submerged fermentation. Incase of substrate 3 differnt substrate with were tested alone. Substrate inulin alone gave 82 U/ml by submerged and 88 U under substrate fermentation. Likewise garlic alone 220 U by substrate fermentation and 248 U. Another substrate corn cob 96 U among substrate and 112 U for submerged condition. Materials are basically composed by cellulose, hemicellulose, lignin, starch, pectin and other fibers are reported as best source for enzyme production (Singhania et al.,2009)

Bacteria do not show an inulinase yield comparable to that control nutrient medium. This means that the substrate enhanced the enzyme productivity but concentrations of carbon on substrate alterd the activity. in this concept it has been well established that garlic+corn cob have higher inulin substrate concentration showed higher enzyme activity. Sharma et al. (2006) reported that the carbon source has been estimated as a major cost factor in enzyme production.

Table 1. Colony morphology of bacterial isolates from goat hair compost

|  |  |  |
| --- | --- | --- |
| CODE | COLONY MORPHOLOGY | PIGMENTATION |
| OS1 | Brick red entire, circular,  opaque, flat, undulate | red |
| OS 2 | White, alrge irregular,Rhizoidal  opaque, convex, curled. | Yellow |
| OS 3 | White, filamentous, opaque | - |
| OS 4 | Creamy white, transluscent small, irregular, opaque, entire.  curled. | green |
| OS 5 | White, translucent. puctiform  opaque, raised, | - |

Table 2.Physiological properties of isolates

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| CODE | KOH  TEST | Inulinase | CATALASE | OXIDASE |
| OS1 | + | - | + | + |
| OS 2 | + | - | + | + |
| OS 3 | - | - | - | + |
| OS 4 | + | + | - | + |
| OS 5 | - | - | + | + |

Table 3. Total protein of cell free extract

|  |  |  |  |
| --- | --- | --- | --- |
| Code | Substrate | Con  mg/L | Enzyme activity  Units |
| SMF 1 | Inulin | 14 | 82 |
| SMF2 | cob | 22 | 122 |
| SMF 3 | garlic | 36 | 248 |
| SMF 4 | cob+inulin | 98 | 580 |
| SMF5 | garlic+inulin | 112 | 660 |
| SMF6 | garlic+cob | 118 | 692 |
| SUBF1 | Inulin Sub F | 16 | 88 |
| SUBF2 | Garlic Sub F | 28 | 220 |
| SUBF3 | Corn cob SubF | 22 | 96 |

**mg/L**

**Figure 1. Total protein of culture filtrate Substrate fermentation**

30 ~~28~~

25

22

20

16

15

Series1

10

5

0

Inulin SubF

Garlic SubF

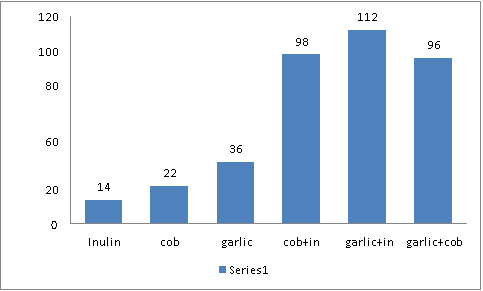
Corn cob SubF

SUBF1

SUBF2

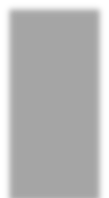
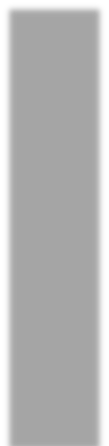
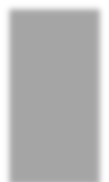
SUBF3

**Mg/Ml**

****

**Figure 2. Total protein of culture filtrate**

**UNITS**



250

220

200

150

100 88

96

Series1

50

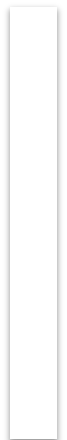
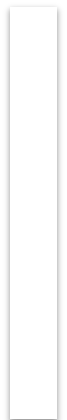
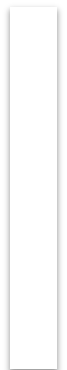
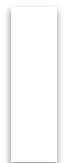
0

Inulin SubF

Garlic SubF

Corn cob SubF

Figure 3.Inulsinase assay substrate fermentation



800

692

700

660

600

580

500

400

300

248

200

122

SMF 1

SMF2 SMF 3

SMF 4 SMF5

SMF6

100

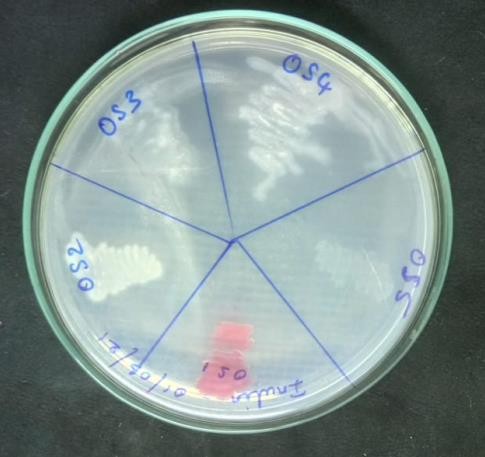
82

0

SMF 1 SMF2 SMF 3 SMF 4 SMF5 SMF6

Figure 4.Inulsinase assay submerged fermentation

Plate 1. Isolation of bacteria from rhizoplane



Collected Onion plant Isolated bacteria on Nutrient agar

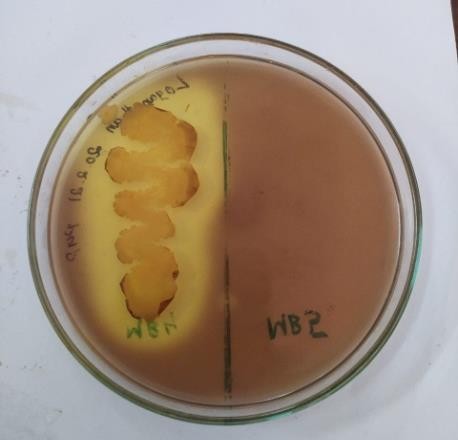


Plate 2 inulinase screening using iodine

Plate 3. agro waste substrate

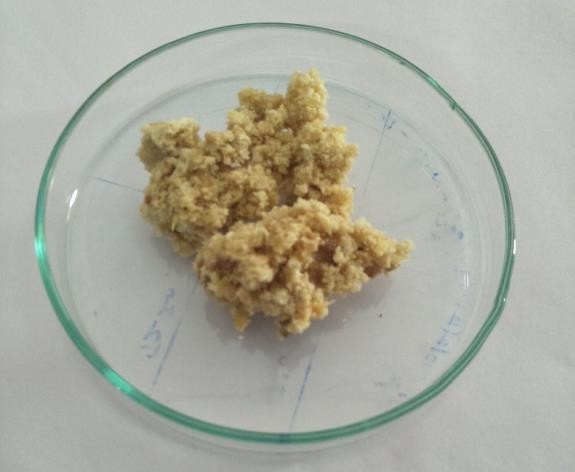


PLATE 4 INULINASE PRODUCTION



Substrate fermentation



Submerged fermentation



Enzyme assay by DNS method

**DISCUSSION AND CONCULUSION**

Inulinase production was evaluated by optimization of substrate and fermentation type. Submerged and substrate medium with good starting extracellular exo-inulinase producer were selected. Extracellular inulinase from Pseudomonas sp was isolated from rhizoplane soil . ferementation medium is prepared in the presence of 1% inulin along with and without substrate during for submerged fermentation. 6 groups of Subf and 3 SSF were evaluated. Among six different submerged fermentation maximum enzyme activity was recorded in garlic+cob. But incase of substrate fermentation significant to moderate enzyme activity was noted on with garlic and cob. Though the activity was comparatively low the correlation of total protein content reveals low protein higher activity in substrate and high protein low activity in submerged activity. Hence the data cocncludes Solid-state fermentation (SSF) is an alternative technique to re-use of agro-industrial and/or sub-products as substrate support for biotechnological production of low to high value biomolecules. It has several environmental advantages, mainly lower capital and operating costs; reduced downstream processing and reduced stirring.

REFERENCES

* 1. Ayyachamy M, Khelawan K, Pillay D, Permaul K, Singh S (2007) Production of inulinase by Xanthomonas campestris pv phaseoli using onion (Allium cepa) and garlic (Allium sativum) peels in solid state cultivation. Lett Appl Microbiol 45(4):439–444
  2. Bellon-Maurel V, Orliac O, Christen P. Sensors and measurements insolid state fermentation: a review. Process Biochem 2003;38:881–96
  3. Couri S, Terzi SC, Pinto GAS, Freitas SP, Costa ACA. Hydrolyticenzyme production in solid state fermentation by Aspergillus niger 3T5B8. Process Biochem 2000;36:255–61
  4. Di Luccio M, Capra F, Ribeiro NP, Vargas GD, Freire D, Oliveira D.Effect of temperature, moisture, and carbon supplementation on lipaseproduction by solid-state fermentation of soy cake by
  5. Dilipkumar M, Rajasimman M, Rajamohan N (2013b) Enhanced inulinase production by Streptomyces sp . in solid state fermentation through statistical designs. 3 Biotech 3(6):509–515
  6. Farinas C.S. Developments in solid-state fermentation for the production of biomass degrading enzymes for the bioenergy sector Renewable and Sustainable Energy Reviews, 52 (2015), pp. 179-188
  7. Figueiredo-Ribeiro RCL,Pessoni Ra And Braga MR.2007. Inulinases produced by microbes from the brazilian cerrado: characterization and potential uses. In: Shiomi N, Benkeblia N and Onodera S (Org), Recent advances in fructooligosaccharides research. 1st ed., Kerala: research signpost press 18: 339-356.
  8. Gencheva P, Dobrev G, Delchev N, Hristov J, Ivanova V (2012) Jerusalem Artichoke and Pea Hulls Based Substrates as Raw Material for Ethanol Production by *Saccharomyces cerevisiae.* IRECHE 4:84–90
  9. Gill P, Manhas R, Singh J And Singh P. 2006. Purification and properties of a heat-stable exoinulinase isoform from Aspergillus fumigatus. J Food Eng 76: 369–375.
  10. Hume MP. Prebiotic supplementation improves appetite control in children with overweight and obesity: a randomized controlled trial. Am J Clin Nutr. 2017;105(4):790-799. doi:10.3945/ajcn.116.140947
  11. Johri BN, Sharma A, Virdi JS. Rhizobacterial diversity in India and its influence on soil and plant health. Adv Biochem Eng Biotechnol. 2003;84:49–89
  12. Kalyani Nair, K.; Kharb, Suman; Thompkinson, D. K. (18 March 2010). "Inulin Dietary Fiber with Functional and Health Attributes—A Review".Food Reviews ternational. 26 (2): 189–203.
  13. Kaur N. and A. GuptaApplications of inulin and oligofructose in health and nutritionJournal of Biosciences, 27 (2002), pp. 703-714
  14. Kays, Stanley J.; Nottingham, Stephen F. (2007-08-13). Biology and Chemistry of Jerusalem Artichoke: Helianthus tuberosus L. CRC Press. ISBN 978-1-4200-4496-6.
  15. Liu XY, Chi Z, Liu GL, Wang F, Madzak C, Chi ZM (2010) Inulin hydrolysis and citric acid production from inulin using the surface-engineered Yarrowia lipolytica displaying inulinases. Metab Eng 12:469–476
  16. Neagu C, Bahrim G (2011) Inulinases-a versatile tool for biotechnology. Innov Rom Food Biotechnol 9:1–11
  17. Niness, K. R. (July 1999). "Inulin and oligofructose: what are they?". The Journal of Nutrition. 129 (7 Suppl): 1402S–6S. doi:10.1093/jn/129.7.1402S. PMID 10395607.
  18. Pandey A, Soccol CR, Selvakumar P, Soccol VT, Krieger N, Fontana JD (1999) Recent Developments in Microbial Inulinases: Its Production, Properties, and Industrial Applications. ApplBiochemBiotechnol 81:35–52
  19. Pandey A. Solid statefermentation. Biochem Eng J 2003;13:81–4. Penicillium sim- plicissimum. Appl Biochem Biotech 2004;113(1– 3):173–80.
  20. Petrova P, Velikova P, Popova L, Petrov K (2015) Direct conversion of chicory our into L (+) -lactic acid by the highly effective inulinase producer Lactobacillus paracasei DSM 23505. Bioresour Technol 186:329–333
  21. Prabhjeet singh and Prabhjot Kaur Gill. (2006) Production of Inulinases Recent advances. Food Technol Biotech. 44(2) :151-162.
  22. Qiu, Y., Zhu, Y., Zhan, Y., Zhang, Y., Sha, Y., Xu, Z., et al. (2019b). Systematic unravelling of the inulin hydrolase from Bacillus amyloliquefaciens for efficient conversion of inulin to poly-(γ-glutamic acid). Biotechnol. Biofuels 12, 1–14. doi: 10.1186/s13068-019-1485-9
  23. Ricca E, Calabro V, Curcio S and Loiro G. 2007. The state of the art in the production of fructose from inulin enzymatic hydrolysis. Crit Rev Biotechnol 27: 129-145
  24. Selvakumar P, Pandey A. Solid state fermentation for the syntesis of inulinase fromStaphylococcusp. and Kluyveromyces marxianusPro-cess Biochem 1999;34:851–8.
  25. Selvakumar P, Pandey A. Solid state fermentation for the syntesis of inulinase fromStaphylococcusp. and Kluyveromyces marxianusPro-cess Biochem 1999;34:851–8.
  26. Sharine Navraj, Mobashshera Tariq, Aruna K (2016) Optimization of Inulinase Production by Stenotrophomonas Maltophila D457 Isolated from Rhizosphere Soil of Musa Acuminata using Garlic Extract International Journal of Research Studies in Microbiology and Biotechnology (IJRSMB) 2,1: 1-14
  27. Sharma AD, Kainth S, Gill PK (2006) Inulinase production using garlic (Allium sativum) powder as a potential substrate in Streptomyces sp. J Food Eng 77(3):486–491
  28. Singh P, Gill PK (2006) Production of Inulinases: Recent Advances. Food Technol Biotechnol 44:151– 162
  29. Singh, R. S., Singh, T., Hassan, M., and Kennedy, J. F. (2020a). Updates on inulinases: structural aspects and biotechnological applications. Int. J. Biol. Macromol. 164, 193–210. doi: 10.1016/j.ijbiomac.2020.07.078
  30. Singhania R.R., Patel A.K., Soccol C.R., Pandey ., A. Recent advances in solid-state fermentation Biochemical Engineering Journal, 44 (2009), pp. 13-18
  31. Vijayaraghavan K, Yamini D, Ambika V and Sowdamini NS. 2009. Trends in inulinase production – a review. Crit Rev Biotechnol 29: 67-77.
  32. Zhao CH, Zhang T, Li M, Chi ZM (2010) Single cell oil production from hydrolysates of inulin and extract of tubers of Jerusalem artichoke by Rhodotorula mucilaginosa TJY15a. Process Biochem 45(7):1121–1126
  33. Zhao CH, Zhang T, Li M, Chi ZM (2010) Single cell oil production from hydrolysates of inulin and extract of tubers of Jerusalem artichoke by *Rhodotorula mucilaginosa* TJY15a. Process Biochem 45:1121–1126