ISSN : 0974 - 7427 Volume 7 Issue 1

Trade Science Inc.

BIOCHEMISTRY
An Indian Journal

Regular Paper

BCAIJ, 7(1), 2013 [22-29]

Optimization studies for biosynthesis of exo-inulinase using chicory waste by *Acinetobacter calcoacitecus*

Edwin A.Pithawala*, N.K.Jain, H.A.Modi

Department of Life Sciences, University School of Sciences, Gujarat University, Ahmedabad, Gujarat, (INDIA)

E-mail : edwinpithawala@gmail.com

ABSTRACT

Biosynthesis of exo-inulinase was investigated from Chicory root tubers. 3 bacterial species were isolated on synthetic medium containing inulin as a sole carbon source. On the basis of maximum exo-inulinase activity on inulin, one of them was selected and identified as *Acinetobacter calcoacitecus*. Incubation of *A. calcoacitecus* was carried for 48 hours, 0.3 gm% pure Inulin as substrate concentration as well as 1.5 gm% concentration of Chicory powder in the growth medium. Optimizations at pH 5.0 and at 37°C were the best conditions for exo-inulinase production. The extracellular enzyme activity was found consistent with the submerged culture method used for biosynthesis of exo-inulinase. The overall production reached up to 1.443 U/ml after 48 hours. The resultant inulinase showed high saccharolytic activity on chicory roots. The isolate which was found commercially important was focused for inulinase production, fructose production and bioremediation approaches. The industrial need of such specific enzymes and improvements required to maximize their bioremediation application in future. Various agro-wastes containing inulin could be economically hydrolyzed with *Acinetobacter calcoacitecus*inulinase into fructose, which has industrial applications besides the beneficial environmental impact by the bioremediation of such agro-wastes.

KEYWORDS

Optimization;
Exo-inulinase;
Chicory;
Acinetobactercalcoaceticus;
Bioremediation.

INTRODUCTION

Inulinase belong to the group of fructanohydrolases, which act as β-fructosidases and hydrolyze inulin to liberate fructose units in a single step from the non-reducing end of fructose chains, some few liberating oligosaccharides as primary products of hydrolysis[10,19]. Inulin is hydrolyzed by 2 types of Inulinases: exo- inulinase (β -D-fructanfructohydrolase) and endo-inulinase (2, 1-β-D-fructanfructanohydrolase)[17,18]. Inulinases are used in the production of high fructose syrups[3], for the production of inulo-oligosaccharides – low caloric saccharides acting as growth factors for beneficial microorganisms in the intestinal flora[20], and is used for production of ethanol from inulin[3].

EXPERIMENTAL

Strain and media

Acinetobacter calcoaceticus strain was isolated

from soil collected from Chicory farms, Vadtal, Gujarat, India. The stain was isolated using Inulin Agar medium. The culture was maintained every 20 days by sub-culturing method using Inulin Agar medium. The composition of formulated Inulin Agar medium includes Inulin (0.5), NaCl (0.5), $MgCl_2$(0.2), K_2HPO_4 (0.2), Chicory root powder (0.5), Agar-agar powder (3.0) g % respectively. For the production of inulinase, natural chicory root powder was used instead of commercial inulin as the sole carbon source to minimize the value of the production cost.

Fatty acid methyl ester (FAME) analysis

Taxonomic status of the organism was ascertained by FAME (Fatty Acid Methyl Ester). All viable & cultivable bacteria which contain C9 – C20 fatty acids can be identified by this method. The identification of the organism is done by comparision with profile library. The culture was grown on TSB Agar for 24hours at 28 °C. 24hours grown sample is transferred in the culture tubes. 1ml of Saponification Reagent is added and subjected to Vortex. Later was kept at 100 °C for 5 and 15 minutes after which it was cooled. Series of reagents viz. 2 ml of Methylation reagent, and 1.25 ml of Extraction Solvent was added with intermediate steps of water bath and cooling. It was put on tumbler Rotospin for 10minutes at 6rpm and aqueous phase which was at the lower side of the culture tube was decanted. 3ml of Base Wash Reagent was added and Rotospin for 5minutes at 6rpm. After which 2/3rd of the upper phase was removed & stored at -20° C in small GC vials[1].

The Gas Chromatography instrument was calibrated by MIDI Fatty Acid Methyl Ester calibration mix containing various C9 – C20 fatty acids after which 2 micro-liter of sample was injected at 25°C into the capillary fused silica column.

Production medium

The strain was cultured on formulated Inulin Agar medium for 48 hours. The composition of optimized fermentation broth is as follows: Chicory root powder - 1.5 gm %, NaCl - 0.5 gm %, $MgCl_2$ - 0.2 gm %, K_2HPO_4 - 0.2 gm %, pH – 5.9.

Growth conditions

*A. calcoacitecus*strain was inoculated in 250 ml Erlenmeyer flasks, each containing 50 ml of sterile production medium. The flasks were incubated with 10% inoculum volume of *A. calcoacitecus* and were incubated under shaking conditions (100 rpm) and under static conditions for crude Inulinase production. Temperature and pH of the medium was optimized during the experiments to achieve maximum enzyme activity and production.

Optimization of various parameters for inulinase production

(a) Time course of inulinase production

Media were taken in 250ml conical flasks & inoculated with 5 ml inoculum of 24 hours old culture. At regular intervals up to 78 hours, the content of the flasks were filtered through Whatman filter paper no.1, the filtrate was used as crude enzyme and inulinase enzyme assay[14], was performed to estimate the inulinase activity.

(b) Effect of inulin concentration on inulinase production

Using pure Inulin (SRL) as a substrate, the different concentrations 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 g % was incorporated each of the 100 ml conical flasks containing 20 ml broth medium and flasks were incubated. Inulinase production was checked after 48 hrs.

(c) Effect of carbon sources on inulinase production

To study the influence of various carbon sources on inulinase production viz. starch, cellulose, mannitol, lactose, glucose, maltose, sucrose, fructose, xylose, inulin and inulin containing different agro-wastes such as Chicory root, coffee seeds (all 0.5 gm %) were incorporated in each of the 100 ml conical flasks containing 20 ml media. Flasks were inoculated with 5 ml inoculums of 24 hours old cultures. Combinations of different carbon sources were also tried.

(d) Effect of agro-waste concentration on inulinase production

Agro-waste used as carbon source was Chicory powder, with various concentrations as 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 g % (w/v) were incorporated in to the medium with an addition of 5 ml of inoculums of 24 hours old cultures and after incubation Inulinase activity was checked after 48 hours.

Regular Paper

(e) Effect of temperature on inulinase production

The effect of temperature on inulinase production was determined in the range of 8 °C to 45 °C. The media were incubated at different temperatures (8 °C, 28 °C, 37 °C, 45 °C & 50 °C) in temperature controlled water bath.

(f) Effect of medium pH on inulinase production

Effects of various pH viz. (3, 4, 5, 6, 7, 8, and 9) of the medium were checked for inulinase production. The initial pH of the medium was 5.9. Flasks were inoculated with 5 ml inoculums of 24 hours old culture.

Bioremediation of chicory wastes using bacterial inulinase

The use of agro-industrial residues as substrate for inulinase bio-production is a good choice to reduce production costs, since enzyme activity will be improved and the downstream step of the process will be viable technically and economically. In addition, the screening of microorganisms that are able to overproduce inulinase using these substrates is fundamental to guarantee successful compatibility with medium constituents[21]. Various agro-wastes such as Coffee Seeds (3%), Agave + Cassava, Coffee + Chicory Seeds, Effluent of Coffee Industries, Formulated Medium, Inulin (Pure) + Formulated Medium were collected in a ratio of 1:3, the pretreated inulin-containing agro-wastes were completed to a volume of 1000 mL and used as a whole in the fermentation medium as a sole source of carbon for inulinase production. The static and shaking conditions were supplied to the fermentation process to obtain maximum inulinase production.

Enzyme assay

Crude enzyme was purified using cheese cloth and centrifuged at 10,000 g for 10 min and supernatant was used as crude Inulinase. 1 ml Enzyme was mixed with 0.3 gm % pure Inulin (SRL) as prepared in 0.1M Acetate buffer (pH- 4.8). The mixture was incubated at 50°C for 10 minutes and the reducing sugar was determined by 3, 5-dinitrosalisylic acid method[6]. One unit of Inulinase activity is defined as the amount of enzyme necessary to hydrolyze 1 µ mol /$min^{-1}$of fructose at 50°C temperature and pH 4.8.

Inulinase activity

Inulinaseactivity[14] was calculated using the formula as:

$$A = C_0 - C_c / 180vt$$

Where, A = Inulinase activity (U/ml.), C_0 = reducing sugar formed after Inulin hydrolysis, C_c = reducing sugar present in control sample, v = volume of liquid culture, t = time of hydrolysis, 180 = molecular weight of fructose.

RESULTS AND DISCUSSION

FAME analysis

The results of FAME analysis reveal that the isolate which was collected from Chicory farms, Vadtal, Gujarat, India, on Inulin agar medium was identified as *Acinetobactercalcoacitecus*. TABLE 1 represents the data analysis of *A.calcoacitecus.*

Volume: DATA1; File: E09C226.71A; SampCtr: 3 ID Number: 2000; Type: SampBottle: 2 Method: RTSBA6; Created: 12/22/2009 4:24:43 PM; Sample ID: NT

Matches:

Library	Sim Index	Entry Name
RTSBA6 6.00	0.244	Acinetobacter-calcoaceticus

Time course for inulinase production

The Inulinase production was checked in Inulin broth medium under static condition at 37°C. After 48 hours, the Inulinase activity was found to be highest about 1.443 U/ml (Figure 2). In inulin, the highest production of inulinase was 2.8 U/ml using *Candida pseudotropicalis*. 14.6 U/ml for *C. kefyr*, 18.7 U/ml for *C. pseudotropicalis*, 18.4 U/ml for *Kluyveromyces marxianus var. bulgaricus*, and 14.3 U/ml for *K. fragilis*[13].

Effect of inulin concentration on inulinase production

Using pure Inulin (SRL) as a substrate, the different concentrations (Figure 3) are incorporated in broth medium and Inulinase production is checked after 48 hrs. The highest production of Inulinase was 0.674 U/ml with chicory root (1.5%) and 0.655 U/ml protein using 3.0 % pure Inulin. Further, concentrations of chicory root powder higher than 1.5% was found to repress the Inulinase production. The results show that the enzyme is an inducible enzyme[7].

Regular Paper

TABLE 1 : FAME analysis data.

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
0.6838	295106	0.006	----	6.6490		----	< min rt	
0.6945	1.128E+9	0.021	----	6.7218	SOLVENT PEAK	----	< min rt	
0.7870	158257	0.015	----	7.3516		----	< min rt	
1.3550	740	0.010	1.115	10.9479	Sum In Feature 2	0.47	ECL deviates -0.005	unknown 10.9525
1.5938	7558	0.010	1.059	12.0004	12:0	4.59	ECL deviates 0.000	Reference 0.004
1.7263	725	0.010	1.035	12.5025	unknown 12.502	----	ECL deviates 0.000	
1.9168	2611	0.010	1.007	13.2031	12:0 2OH	1.51	ECL deviates -0.001	
1.9986	4143	0.009	0.997	13.4831	12:0 3OH	2.37	ECL deviates 0.000	
2.1495	5997	0.010	0.980	13.9999	14:0	3.37	ECL deviates 0.000	Reference -0.002
2.3089	742	0.011	0.965	14.5170	unknown 14.502	----	ECL deviates 0.001	
2.3439	486	0.009	0.962	14.6305	15:0 iso	0.27	ECL deviates -0.002	Reference -0.005
2.4585	1191	0.009	0.953	15.0022	15:0	----	ECL deviates 0.002	
2.5132	415	0.010	----	15.1741		----		
2.6225	8916	0.009	0.941	15.5175	Sum In Feature 2	4.82	ECL deviates 0.002	14:0 3OH/16:1 iso I
2.6402	900	0.009	0.940	15.5731	16:0 N alcohol	0.49	ECL deviates -0.001	
2.7246	28137	0.009	0.935	15.8381	Sum In Feature 3	15.10	ECL deviates -0.002	16:1 w7c/16:1 w6c
2.7764	44264	0.009	0.933	16.0010	16:0	23.69	ECL deviates 0.001	Reference -0.004
2.9791	501	0.011	0.924	16.6364	17:0 iso	0.27	ECL deviates -0.001	Reference -0.006
3.0362	2516	0.010	0.922	16.8155	17:1 w8c	1.33	ECL deviates 0.000	
3.0678	1121	0.010	0.920	16.9145	17:0 cyclo	0.59	ECL deviates 0.000	
3.0957	2338	0.009	0.920	17.0019	17:0	1.23	ECL deviates 0.002	Reference -0.004
3.2109	790	0.009	----	17.3649		----		
3.2240	1721	0.011	----	17.4059		----		
3.2856	989	0.009	0.915	17.5999	18:3 w6c (6,9,12)	0.52	ECL deviates 0.000	
3.3224	1647	0.012	----	17.7158		----		
3.3469	53889	0.010	0.913	17.7931	18:1 w9c	28.25	ECL deviates -0.001	
3.3629	14389	0.009	0.913	17.8433	Sum In Feature 8	7.54	ECL deviates -0.004	18:1 w7c
3.3905	560	0.011	0.913	17.9304	18:1 w5c	0.29	ECL deviates -0.007	
3.4123	5882	0.009	0.912	17.9989	18:0	3.08	ECL deviates -0.001	Reference -0.007
3.4506	1042	0.014	----	18.1227		----		
3.5266	814	0.015	----	18.3684		----		
3.5433	386	0.009	----	18.4223		----		
3.6325	4594	0.010	----	18.7104		----		
3.7026	425	0.010	0.911	18.9370	19:0 cyclo w8c	0.22	ECL deviates 0.005	
3.9351	8329	0.010	----	19.7068		----		
4.2284	11503	0.010	----	20.6751		----	> max rt	
----	9655	---	----	----	Summed Feature 2	5.29	12:0 aldehyde ?	unknown 10.9525
----	-----	---	----	----		----	16:1 iso I/14:0 3OH	14:0 3OH/16:1 iso I
----	28137	---	----	----	Summed Feature 3	15.10	16:1 w7c/16:1 w6c	16:1 w6c/16:1 w7c
----	14389	---	----	----	Summed Feature 8	7.54	18:1 w7c	18:1 w6c

ECL Deviation: 0.002; Reference ECL Shift: 0.005; Number Reference Peaks: 7; Total Response: 206101 Total Named: 186362; Percent Named: 90.42%; Total Amount: 176873

Regular Paper

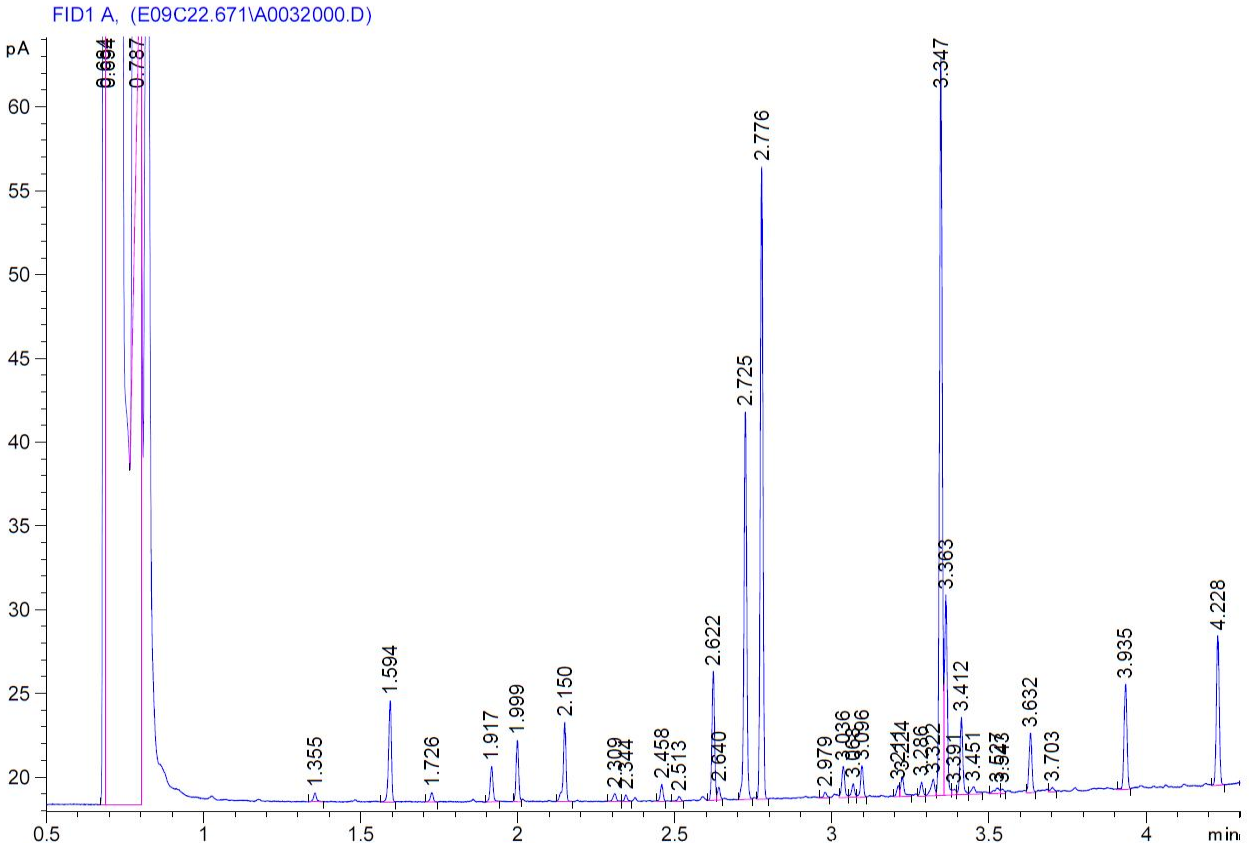

Figure 1 : FAME analysis graph of acinetobactercalcoacitecus.

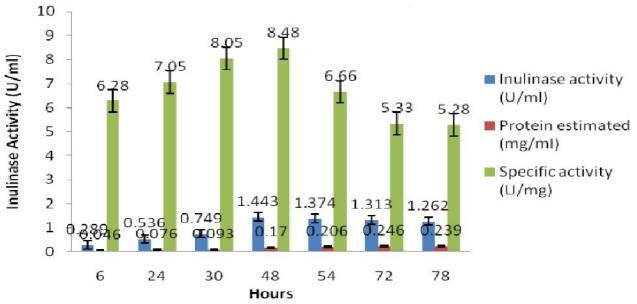

Figure 2 : Production curve for *Acinetobacter calcoacitecus.*

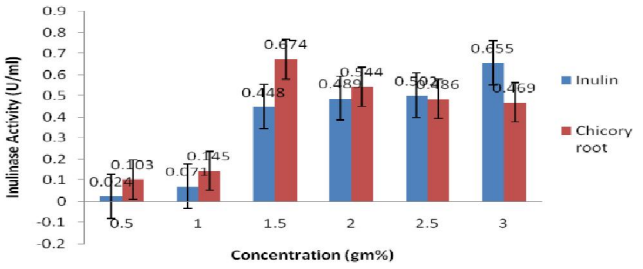

Figure 3 : Effect of substrate (pure inulin) on enzyme production.

Effect of carbon sources on inulinase production

It has been reported that different carbon sources have significant influences on inulinase production[16]. A number of carbon sources (Figure 4) was used as substrate with fixed concentration of 1.5 gm% incorporated into the medium and Inulinase production was checked after 48 hours. The highest production of Inulinase using Inulin and Fructose was 0.546 U/ml and 0.376 U/ml activities respectively. The activity was obtained as 0.246 U/ml with Chicory root powder. Indeed, as shown in Figure 4, inulinase production by *Acinetobactercalcoacitecus* was also influenced greatly by different carbon sources in the medium.

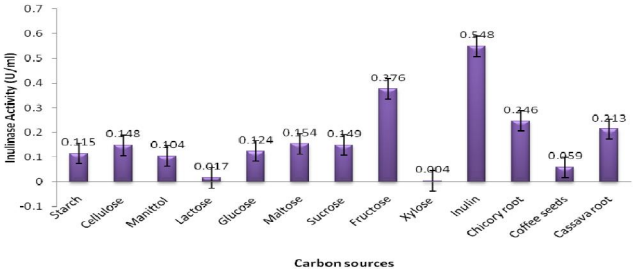

Figure 4 : Effect of carbon sources on Inulinase production.

It can be seen clearly from Figure 4 that inulin was the best carbon source for inulinase production, and fructose was the second better source for it. However, the lowest inulinase was produced in the medium containing xylose. This may be due to xylose repression on

inulinase synthesis in the cells. Other different carbon sources used had no notable influences on cell growth of the *Acinetobacter calcoacitecus* (Figure 4).

Effect of agro-waste concentration on inulinase production

Recently, a number of research projects have been focused on the use of agricultural wastes as substrate for the production of commercially important enzymes[5]. Agro-waste used as carbon source was Chicory powder, with various concentrations (Figure 5) and Inulinase activity was checked after 48 hours. The higher Inulinase activity was obtained as 0.843 U/ml using chicory powder in 1.5 gm % concentration.

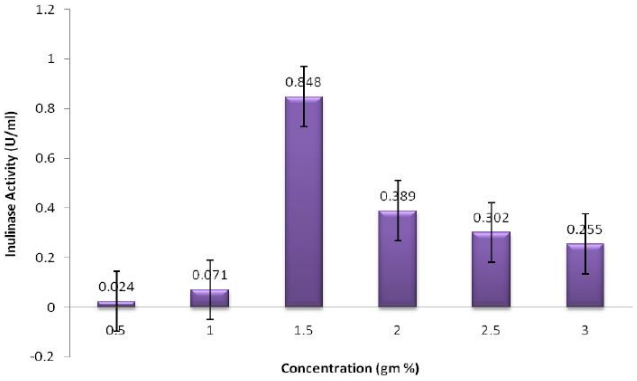

Figure 5 : Effect of chicory concentration on inulinase production.

Effect of temperature on inulinase production

The Inulin broth flasks are incubated at different temperatures (Figure 6) and Inulinase production after 48 h using Pure Inulin and Chicory powder. The optimum temperature was found to be 37°C and 45°C using Chicory and pure Inulin as substrates respectively. Cho and Yun[20] have shown that Xanthomonasoryzae produce an endoinulinase having optimum temperature of 50° C whereas Abeer[4] indicated that Streptomyces griseus produces an inulinase having optimum temperature of 40° C. F. oxysporum[3], Penicilliumjanczewskii[2] and A. niger[3] produce inulinases optimal at 30…40ÚC, indicating that the optimal temperatures of inulinases from different species of microorganisms are significantly different.

Effect of medium pH on inulinase production

The Inulin broth flasks containing media are prepared at different pH (Figure 7) and the Inulinase production is checked after 48 hrs the activity was calculated as 0.832 U/ml using Inulin and 0.164 U/ml using chicory powder.

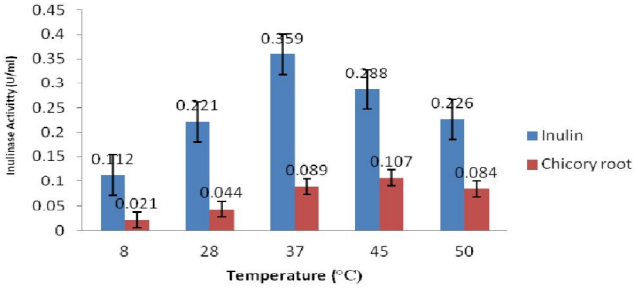

Figure 6 : Effect of temperature on inulinase production.

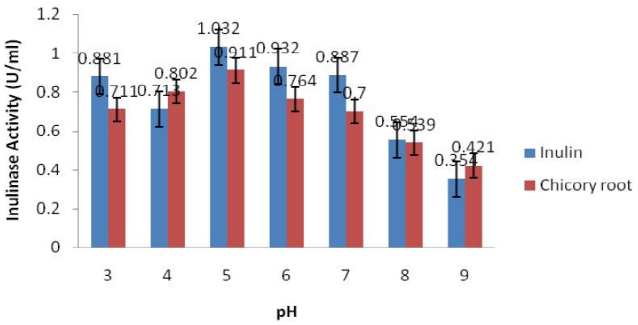

Figure 7 : Effect of medium pH on inulinase production.

The optimum medium pH was found to be 5.0 using Inulin and chicory powder as substrates. This low pH could be advantageous in the industrial preparation of sugar syrups because at low pH, there is reduced colour formation which is desirable[15]. Inulinases from *A. niger* were found to have an optimum pH at 4.4, inulinases produced by *A. versicolor* have the optimal pH at 5.5 and *Penicilliumj-anczewskii* produces inulinases with optimal pH at 4.8-5.0[2]. Inulinases produced by *A. ochraceus* are optimal at pH 4.5[11]. There are inulinases with lower optimal pH: K. marxianus produces inulinases which have the highest activity at 3.5[12] and *Pichiaguilliermondii* at 3.4[9].

Bioremediation of agro wastes using bacterial inulinase

The inulinase activity was found to be consistent with various Argo wastes used (TABLE 2). In the bioremediation of such agricultural by-products by *Acinetobactercalcoacitecus*,inulinase introduces feasible benefit trends in various economic and environmental aspects, wherein fructose is produced during the fermentation process[8]. Maximum inulinase activity was found with inulin incorporated in formulated media under static conditions (TABLE 2).

Regular Paper

TABLE 2 : Bioremediation of agro wastes using bacterial inulinase.

Agro wastes	Fructose Production (µgm/ml)		Protein estimation (µgm/ml)		Inulinase Activity (U/ml)		Specific Activity (U/mg)	
Conditions	Static	Shaking	Static	Shaking	Static	Shaking	Static	Shaking
Coffee Seeds	2440	2400	215	203	1.355	1.333	0.0063	0.0065
Agave + Cassava	2562	2443	263	249	1.422	1.357	0.0054	0.0054
Coffee + Chicory Seeds	2623	2508	289	290	1.457	1.393	0.0050	0.0048
Effluent of Coffee Industries	2351	2199	264	208	1.306	1.221	0.0049	0.0058
Formulated Medium	2916	2811	262	257	1.620	1.561	0.0061	0.0060
Inulin (Pure)+ Formulated Medium	3957	3869	249	238	2.195	2.149	0.0088	0.0090

CONCLUSIONS

In this work, optimizations of medium components were achieved to get maximum inulinase production from identified culture *Acinetobactercalcoaceticus*. Among the variables, Inulin containing chicory powder was found to be the most significant variable from other carbon sources. From further optimization studies the optimized values of the variables for inulinase production were as follows: chicory powder - 1.5 gm %, Inulin – 3.0 gm %, at 37 ^{0} C temperatures for 48 hours at 5.0 pH. This study showed that the inulin containing chicory waste constitutes a good carbon source for the production of inulinase. Using the optimized conditions, the produced activity reaches 1.443U/ml after 48 hour. The results show a close concordance between the expected and obtained activity level. The present study therefore, highlights the importance of microbial inulinase in the hydrolysis of inulin and production of fructose which can be used in various industries. In future, a process for continuous production of fructose syrups can be developed by immobilization of the extracted enzyme. Besides the production of fructose and exo-inulinase it could also have a beneficial environmental impact by bioremediation of inulin containing agro-wastes as it could serve as the carbon source for the production of exo-inulinase.

REFERENCES

[1] C.Kunitsky, G.Osterhout, M.Sasser; Identification of microorganisms using fattyacid methyl ester (FAME) analysis and the MIDI sherlock microbial identification system, MIDI, Inc., Newark, DE, USA, www.peda.org.bookstore.

[2] D.Sharma, S.Kainth, P.K.Gill; Inulinase production using garlic (Allium sativum) powder as a potential substrate in streptomyces sp., Journal of Food Engineering, **77**, 486-491 **(2006)**.

[3] Pandey, C.Soccol, R.P.Selvakumar, V.T.Soccol, N.Krieger, J.D.Fontana; Recent developments in microbial inulinases: Its production, properties and microbial applications, Applied Biochemistry and Biotechnology, **81**, 35–52 **(1999)**.

[4] F.Abeer; Studies on inulinases produced by some bacteria. M.Sc.Thesis, Tanta University, **(2004)**.

[5] S.Kahraman, O.Yesilada; Industrial and agricultural wastes as substrates for laccase production by white-rot fungi, Folia Microbiology, **46**, 133-136 **(2001)**.

[6] G.L.Miller; Use of dinitrosalicylic reagent for the determination of reducing sugars, Analytical Chemistry, **31**, 426-428 **(1959)**.

[7] J.W.Yun, C.H.Song, J.W.Choi, S.K.Song; Production of inulo-oligosaccharides from inulin by recombinant *E. coli* containing endoinulinase activity, Bioprocess Engineering, **21**, 101–106 **(1999)**.

[8] J.W.Yun, D.H.Kim, B.W.Kim, S.K.Song; Biotechnology Letters, **19(6)**, 553-556 **(1997)**.

[9] L.Gao, Z.Chi, J.Sheng, L.Wang, J.Li, F.Gong; Inulinase-producing marine yeasts: Evaluation of their diversity and inulin hydrolysis by their crude enzymes, Microbial Ecology, **54**, 722-729 **(2007)**.

[10] L.Zittan; Enzymatic hydrolysis of inulin: An alternative way to fructose production. Starch, **33**, 373-377 **(1981)**.

[11] L.H.S.Guimaraes, H.F.Terenzi, M.L.Polizeli, J.A.Jorge; Production and characterization of a thermostable extracellular β-dfructofuranosidase produced by aspergillusochraceus with agroindustrial residues as carbon sources, Enzyme and Microbial Technology, **42**, 52-57 **(2007)**.

[12] M.Mazutti, G.Ceni, M.Luccio, H.Treichel; Production of inulinase by solid-state fermentation: Effect

of process parameters on production and preliminary characterization of enzyme preparations, Bioprocess Biosystemic Engineering, **30**, 297-304 **(2007)**.

[13] M.L.Cazetta, P.M.M.Martins, R.Monti, J.Contiero; Yacon (Polymniasanchifolia) extract as a substrate to produce inulinase by Kluyveromy-cesmarxianus var.bulgaricus, Journal of Food Engineering, **66**, 301–305 **(2005)**.

[14] N.A.Zherebtsov, I.N.Abramova, S.A.Shelamova, T.N.Popova; Identification of catalytically active groups in inulinase from bacillus polymyxa 722, Applied Biochemistry and Microbiology, **39(6)**, 544-548 **(2003)**.

[15] R.C.Patil, D.S.Wavhal, D.Patil, A.Patil, N.Patil, R.R.Tamboli; Activity and applications studies of enzyme inulinase Extracted from bacteria, Plant Archives, **11**, 931-934 **(1994)**.

[16] R.S.Singh, R.Dhaliwal, M.Puri; Production of inulinase from kluyveromycesmarxianus YS-1 using root extract of *Asparagus racemosus*, Process Biochemistry, **41**, 1703–1707 **(2006)**.

[17] S.Baumgartner, W.Praznik; Purification of exo- and endo-inulinase from crude inulinase extract for the analysis of fructans. International Journal of Biological Macromolecules, **2**, 247 **(1995)**.

[18] T. B.Uhm, M.S.Chung, S.H.Lee, F.Gourronc, I.Housen, J.H.Kim, V.Beeumen, B.Haye, J.Vandenhaute; Purification and characterization of *Aspergillusficuum* endoinulinase, Bioscience Biotechnology and Biochemistry, 146-151 **(1999)**.

[19] T.Nakamura, S.Maruki, S.Nakatsu, S.Ueda; Studies on microbial inulase: General properties of an extracellular inulase (Pll) from Aspergillus sp. Journal of Agricultural Chemical Society Japan, **52**, 581-587 **(1978)**.

[20] Y.J.Cho, J.W.Yun; Purification and characterization of an endoinulinase from xanthomonasoryzae on submerged process, Biochemistry, **37**, 1325-1331 **(2002)**.

[21] Y.Makino, H.Treichel, M.A.Mazutti, F.Maugeri, M.I.Rodrigues; Inulinase bio-production using agroindustrial residues: Screening of microorganisms and process parameters optimization, Journal of Chemical Technology and Biotechnology, **84**, 1056-1062 **(2009)**.