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Comparative Study on Production and Purification of Itaconic Acid by *Aspergillus terreus* Utilizing Maize Flour, Corn Starch and Waste Potatoes

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ABSTRACT

Itaconic acid (IA) is an organic acid having a wide scope of applications in different fields like pharmacy, agriculture, dental, ophtalamic and chemistry. In this study, a systematic process optimization was performed with an Aspergillus terreus MTCC 479 by using cheap raw materials like maize flour, waste potatoes and corn starch. Acid hydrolysis was performed by using HCl and Enzymatic hydrolysis was carried out by production of amylase (enzyme activity 126U/ml) using Aspergillus oryzae MTCC 645.Itaconic acid production was 15.5g/l from control (with pure glucose), 10.3g/l from corn starch, 6.5g/l from maize flour and 5.8g/l from waste potatoes at 120h. After purification by solvent extraction method using n-Butanol as solvent, itaconic acid concentration was increased 2-3 times i.e., 40.80g/l for control ,35.75g/l for corn starch, 22.75g/l for maize flour and 17.55g/l for waste potatoes respectively using 1:3 aqueous to organic phase ratio. So this study shows the comparison of production of itaconic acid by using cheap raw materials and also the use of inexpensive method for purification which will be helpful in decreasing the process economics.

Key words: *Aspergillus terreus, Itaconic acid, Aspergillus oryzae, Bromination, Solvent extraction method*

INTRODUCTION

Itaconic acid is an unsaturated dicarboxylic acid which has several industrial applications to be used for the production of Unsaturated Polyester Resins (UPR), Styrene Butadiene rubber, super absorbents, emulsion paints, synthetic fibres, lubricating oil additives and special optical lens[4,18]. Itaconic acid was produced by various chemical methods prior to 1960, but none of them were commercialized due to low yield.

Now most of the itaconic acid is produced by fermentation using *Aspergillus terreus* which accumulate more itaconic acid than *Aspergillus Itaconicus*[2,16]. Although other microorganisms *U.maydis*, *Candida* sp., *Pseudozyma antarctica*, *E.coli* have been reported but maximum production of more than 80g/l has been reported by using *A.terreus*[9,10,15,20]. The pathway of biosynthesis of itaconic acid reveals that it is derived from one of the steps of Tricarboxylic acid(TCA) cycle. Cis-aconitate derived from citrate is converted to Itaconate by Cis aconitate decarboxylase (Cad A) releasing CO_2[18]. Maximum production of itaconic acid is achieved by using glucose as substrate which is very expensive to be used for commercial production of itaconic acid and its current price ranges from $0.35- $0.60/kg[4].

The other raw materials which are comparatively cheaper than glucose like corn syrup, starch, and molasses are already being tested for production of itaconic acid[18]. The present study also demonstrates the production of itaconic acid utilizing maize flour, corn starch and waste potatoes which are much

cheaper than glucose so helps in controlling the process economics. For purification, solvent extraction method is used which is inexpensive method than other methods like Liquid chromatography. Also it is easy to scale up and permits continuous steady state operation.

MATERIALS AND METHODS

Strain and Chemicals

Aspergillus terreus MTCC NO.479 and *Aspergillus oryzae* MTCC NO.645 were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Maize flour, waste potatoes and corn starch were obtained from local market, n-Butanol was received from Fischer scientific (Mumbai) and all other chemicals were of analytical reagent grade.

Culture Media

Aspergillus terreus was grown on Czepak Dox medium containing Sucrose,30(gl^{-1}), Yeast extract 5(gl^{-1}), K_2HPO_4 1(gl^{-1}), $NaNO_3$ 300(gl^{-1}), $MgSO_4.7H_2O$ 50(gl^{-1}), KCl 50(gl^{-1}), $FeSO_4.7H_2O$ 1(gl^{-1}), Agar 15(gl^{-1}) .*Aspergillus oryzae* was propagated on Potato dextrose Agar (Hi-Media) with PDA 24 (gl^{-1}), pH-5.1.Slants were grown at 30^0C for 5 days and stored at 4^0C.

Acid Hydrolysis

Starch estimation in raw materials was done by anthrone method[12] and determination of reducing sugars was done by DNS method[11].Hydrolysis was performed by acid as well as by using amylase enzyme produced from *Aspergillus oryzae*. Acid hydrolysis of three different starchy materials was done by using the Hydrochloric acid. Optimization of acid hydrolysis was done by varying the concentration of Hydrochloric acid as well as by varying the concentration of substrate.

Enzymatic hydrolysis

Hydrolysis was done by production of amylase from *Aspergillus oryzae*. To the 5 days old culture slants, 5ml of 0.9% saline solution along with the 0.1% Tween-80 was added. Spores were dislodged using inoculation loop under sterile conditions. Inoculum was prepared by adding these spores into Potato dextrose broth and keeping this Broth at 30^0C under shaking conditions for 24 hours. This inoculum was further used for production of amylase enzyme in amylase production media: Corn starch 24(gl^{-1}), Yeast extract 36(gl^{-1}), Na_2HPO_4 47(gl^{-1}), KCl 0.2(gl^{-1}), $MgCl_2$ 0.2(gl^{-1}), $CaCl_2$ 1(gl^{-1}).

Effect of incubation period

Effect of incubation period on amylase production was studied by measuring enzyme activity after every 24 h. Culture filtrate was harvested and enzyme assay was performed up to 120h. After maximum production, whole broth was centrifuged at 15000rpm for 30 minutes to extract the enzyme.

Optimization of hydrolysis conditions

Effect of substrate concentration (5%, 10%, 15% w/v) as well as time period (4, 8, 12, 16, 20, 24 h) at 50^0C was studied to get maximum hydrolysis of substrates. After maximum hydrolysis, Centrifugation was done at 10000 rpm for 30 min to extract glucose. The glucose obtained by this method was further used for itaconic acid production.

Enzyme assay

The reaction mixture consisted of 1.25 mL of 1% (w/v) soluble starch (Merck) solution, 0.25 mL of 0.1M sodium acetate buffer (pH 5.0), 0.25 mL of distilled water, and 0.25 mL of properly diluted crude enzyme extract. After 10 min of incubation at 50°C, the liberated reducing sugars (glucose equivalent) were estimated by the dinitrosalicylic acid method of Miller.

One unit of amylase is defined as the amount of enzyme releasing 1µ mol. of glucose equivalent/min under the assay conditions.

Enzyme activity (U/ml) = Concentration obtained from standard graph× Dilution factor ×1000/Time for enzyme incubation× 1g mole of substrate.

Culture conditions for Itaconic acid production

Conidiospores from 7 day old culture slants were suspended in 5ml sterile 0.05 mol/l phosphate buffer (pH 6.5) containing 0.1% Tween-80 and used to inoculate 500ml conical flasks containing 100 ml sterile Czepak Dox medium to give high spore concentration. After incubation on rotary shaker at 200 rev/min for 24 hours at 35^0C, fractions of 10 ml were used to inoculate 90ml sterile production medium; Glucose 100(gl^{-1}), Ammonium sulphate 2.36(gl^{-1}), KH_2PO_4 0.11(gl^{-1}) $MgSO_4.7H_2O$ 2.1(gl^{-1}), $CaCl_2.2H_2O$ 0.13(gl^{-1}), NaCl 0.074(mgl^{-1}), $CuSO_4.5H_2O$ 0.2(mgl^{-1}), $FeSO_4.7H2O$ 5.5(mgl^{-1}), $MnCl_2.4H_2O$ 0.7(mgl^{-1}) and

$ZnSO_4.7H_2O$ ($1.3mgl^{-1}$) in 500 ml conical flasks. Cultures were then incubated for 6 days under the same conditions as above. Samples were taken after every 12 h till 6 days, diluted with deionized water to solubilize the itaconic acid and filtered through 0.2 µm whatmann discs .This samples were analysed for itaconic acid production by Bromination method[5].

Itaconic acid purification

Itaconic acid was purified by solvent extraction method using n-butanol as solvent. Itaconic acid broth was filtered through whatman (0.2µm) filter discs. Aqueous itaconic acid solution was prepared by dissolving itaconic acid in equal amount of deionized water. Then again filtration was done by using whatman (0.2µm) syringe filter. The saturated solution of itaconic acid was mixed with organic solvent (n-Butanol) in different ratios i.e. (1:1, 1:2, 1:3, 1:4) to optimize the volume of extractant for maximum purification. Solutions were mixed properly for 45 minutes by using magnetic stirrer. The mixture was transferred to separating funnel (500ml) and allowed to settle for 1 hour. Two stable phases were formed depending upon the density difference between aqueous phase and organic phase. After the phase separation, volume of aqueous as well as organic phase was measured. The aqueous and organic phases were analyzed for determination of itaconic acid concentration by titration method in different ratios of n-Butanol. Degree of extraction (%E) was calculated[6].

RESULTS AND DISCUSSION

The present study was designed to check the potential of cheap raw materials for production of itaconic acid. Hydrolysis of raw materials was done by acid and enzymatic treatment. Production of itaconic acid was quite significant by using cheap raw materials also. Further purification improved the yield of itaconic acid in an effective way.

To start with the study, the starch content was measured by using anthrone method and found to be 95%, 75% and 16% in corn starch, maize flour, and waste potatoes respectively. The starch content was found to be less in waste potatoes as compared to maize flour and corn starch. It is due to more amounts of dietary fibers, fat content as well as more moisture content.Waste potatoes are affected by environmental conditions as well as certain different microorganisms due to which starch content was decreased.

Effect of substrate concentration on acid hydrolysis

Substrate concentration was varied from 5% to 20 % (w/v). Yield of reducing sugars (g/l) and hydrolysis % (Table 1) was calculated by using standard graph of glucose. It was observed that substrate concentration affects the hydrolysis of raw materials in an effective way (Table 1). In case of maize flour, maximum yield of reducing sugars 60g/l and 80 % hydrolysis was observed for 10% substrate concentration. In case of Corn starch, maximum yield of reducing sugars 42.5g/l and 57% hydrolysis for 10%(w/v) substrate concentration was observed. While in case of waste potatoes, maximum yield of reducing sugars 17.5g/l and 73% hydrolysis was observed for 15%(w/v) substrate concentration. It is due to fact that more the quantity of starch granules, less is the chance of penetration of hydrogen ions into amorphous regions, leading to less degradation of amylose and amylopectin[1].

Table 1. Effect of substrate concentration on yield of reducing sugars as well as hydrolysis (%) by acid hydrolysis method

Substrate	Substrate concentration	Absorbance	yield of reducing Sugar	Hydrolysis
	(%)	(540nm)	(g/l)	(%)
Maize flour	5	0.51	21.5	58
	10	**1.41**	**60**	**80**
	15	1.32	55	50
	20	1.25	52.5	35
Corn starch	5	0.45	20	47
	10	**1.01**	**42.5**	**57**
	15	0.76	32.5	30
	20	0.70	30	20
Waste potatoes	5	0.1	5	62
	10	0.24	11	68
	15	**0.41**	**17.5**	**73**
	20	0.35	15.5	49

Effect of acid concentration on acid hydrolysis of substrates

Acid concentration was also varied in the ratio 1:0.5 to 1:5(substrate concentration: acid concentration) for the 10% substrate concentration in case of maize flour and corn starch while 15% substrate concentration for waste potatoes.(Table 1). Wang *et al.*,[17] had found that both amylose and amylopectin were hydrolyzed and the extent of hydrolysis was affected by the acid concentration.

For all the three substrates, 1:1 ratio of substrate concentration to acid concentration gave best results (Table 2). It gave maximum yield of reducing sugars 60g/l, 42g/l, 17g/l and hydrolysis 80%, 56%, 72% in case of maize flour, corn starch, waste potatoes respectively. In case of all the starch materials, significant effect up to a certain concentration of acid i.e. when acid concentration equivalent to the substrate concentration was observed. After this equivalent ratio, as the acid concentration increased more than that of the substrate, yield of reducing sugars as well as hydrolysis got decreased for all raw materials.

Table 2. Effect of Acid concentration on yield of reducing sugars as well as Hydrolysis (%)

Substrate	HCl concentration (S:A Ratio)	Absorbance (540nm)	yield of reducing Sugar (g/l)	Hydrolysis (%)
Maize flour	1:0.5	0.98	42	56
	1:1	**1.41**	**60**	**80**
	1:3	1.01	55	73
	1:5	1.1	47.5	63
Corn starch	1:0.5	0.82	34	35
	1:1	**1.21**	**42**	**56**
	1:3	0.9	38	40
	1:5	0.85	35	37
Waste potatoes	1:0.5	0.25	12.5	52
	1:1	**0.41**	**17**	**72**
	1:3	0.34	15	63
	1:5	0.29	13	53

This is due to the fact that high concentration of acid caused the reducing sugar degradation. The soft hydrolysis conditions led to a sugar-rich pre-hydrolysate. When using harsh pretreatment conditions, sugar recovery in raw materials decreased.

Effect of Incubation period on amylase production used for enzymatic hydrolysis

The effect of incubation period on enzyme production was also observed by varying the time period for production of amylase from 1st to 6th days (Fig. 1). On 4th day, enzyme activity was maximum up to 126U/ml. The enzyme activity first increased with increasing time period i.e. it increased up to 4th day and then started decreasing till 6th day.

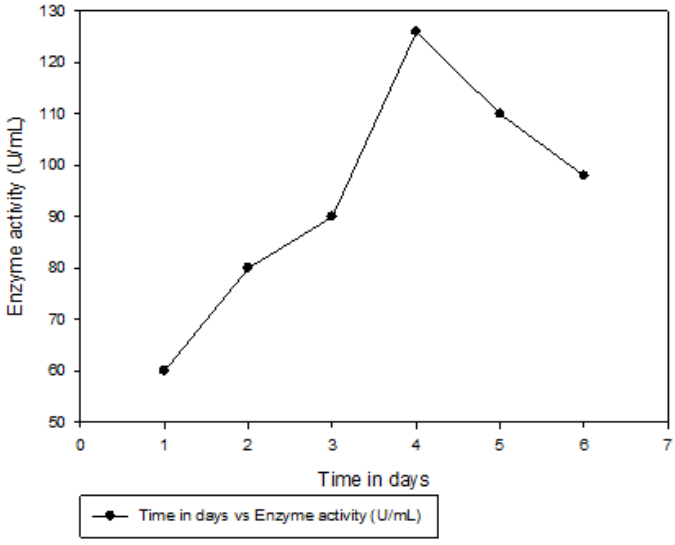

Fig.1: Effect of incubation period on enzyme production

The incubation period is directly related with the production of enzyme and other metabolic process up to a certain extent. Further increase in incubation time decreases the enzyme production. It might be due to deficiency of nutrient, accumulation of toxic substances[3,14].

Optimization of conditions for hydrolysis by amylase

After the production, the amylase enzyme was extracted from fermentation broth by centrifugation and used further for hydrolysis of maize flour, Corn starch and waste potatoes (Fig. 2). Fig. 2 showed that, for all three substrates, hydrolysis percentage was highest at 20h for 10% (w/v) substrate concentration. Maximum hydrolysis was found to be in case of maize flour i.e. 88%. For corn starch and waste potatoes, hydrolysis was found to be 84% and 67% respectively. Enzymatic hydrolysis method produced maximum reducing sugars as compared to acid hydrolysis method (Table 1,2,3), so glucose produced by this enzymatic hydrolysis method was further used for production of itaconic acid. For every substrate, yield of reducing sugars and hydrolysis increased up to 20h and after that it became stable. 10% (w/v) substrate was found to be optimized to get the maximum hydrolysis for all the three starchy materials (Table 3). Stable glucose production after 10% (w/v) substrate is due to enzyme inhibition by the presence of impurities. Moreover, high concentration of substrate might reduce the water content in reaction mixture which lowered pentose yield and also lowered the rate of hydrolysis[19] as shown in hydrolysis progress in Fig. 2.

Table 3. Optimization of time period and substrate concentration for maximum hydrolysis (%) by enzymatic method

Time (Hours)	Maize flour			Corn starch			Waste potatoes		
	5%	10%	15%	5%	10%	15%	5%	10%	15%
4	47	37	20	31	26	20	31	46	25
8	62	50	25	43	38	25	46	56	35
12	68	69	34	47	55	30	56	58	36
16	82	80	49	52	67	36	63	63	46
20	85	**88**	61	67.5	**84**	47	65	**67**	55
24	84	86	61	67	83	46.6	65	66	53

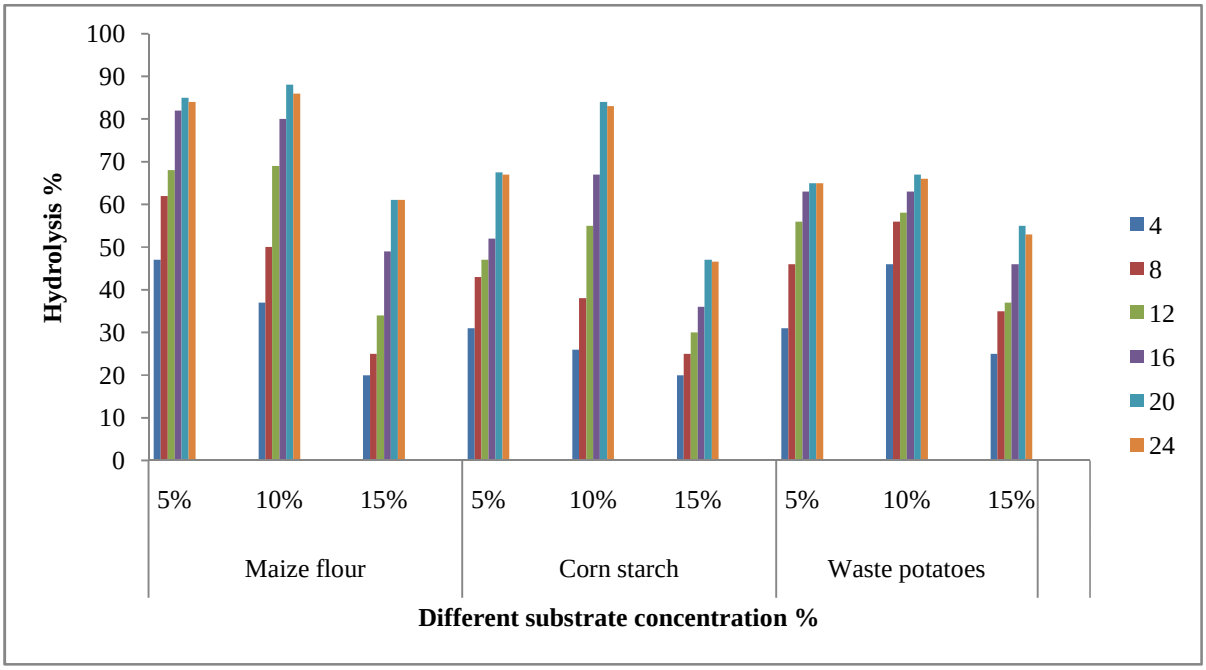

Fig. 2: Optimization of time period and substrate concentration for hydrolysis % by amylase

Itaconic acid Production

The glucose released after hydrolysis of starchy materials was used for the production of itaconic acid. All the ingredients were added which are necessary for the growth of *A. terreus* and production of itaconic acid. To compare the production of itaconic acid, pure glucose was used in control. Samples were taken after every 24h and analysis of itaconic acid was done by Bromination method.

For maize flour, production of itaconic acid was 6.5g/l while in case of waste potatoes and corn starch, production was 5.8g/l and 10.3g/l respectively. (Fig.3) Production of itaconic acid was increasing with time period from 24 to 120 h for all raw materials (Table 4). Maximum production of itaconic acid i.e. 15.5g/l was in control in which pure glucose was used as raw material. Besides that considerable production was found to be in case of corn starch i.e.10.3g/l which is due to amylolytic activity shown by *A. terreus*. These results are supported by the studies done by Petruccioli *et al*.,[13] and Kirimura *et al.*,[7] that there is presence of strong amylolytic activity in *Aspergillus terreus* which hydrolysed rest of the liquefied corn starch left after the acid and enzymatic hydrolysis. As corn starch got completely liquefied after autoclaving of medium so *A. terreus* showed more amylolytic activity in case of liquefied corn starch, so production of itaconic acid was comparatively more in case of corn starch.

Table 4. Comparison of Itaconic acid production as estimated by Bromination method

Time (hours)	Itaconic acid production(g/l)			
	Control	Maize flour	Corn starch	Waste potatoes
24	0.52	0.23	0.48	0.12
48	1.32	0.96	1.2	0.85
72	6.53	3.39	5.42	2.4
96	11.3	5.8	9.6	4.9
120	**15.5**	**6.5**	**10.3**	**5.8**
144	14.6	5.9	9.52	4.5
168	13.24	4.52	8.35	3.69

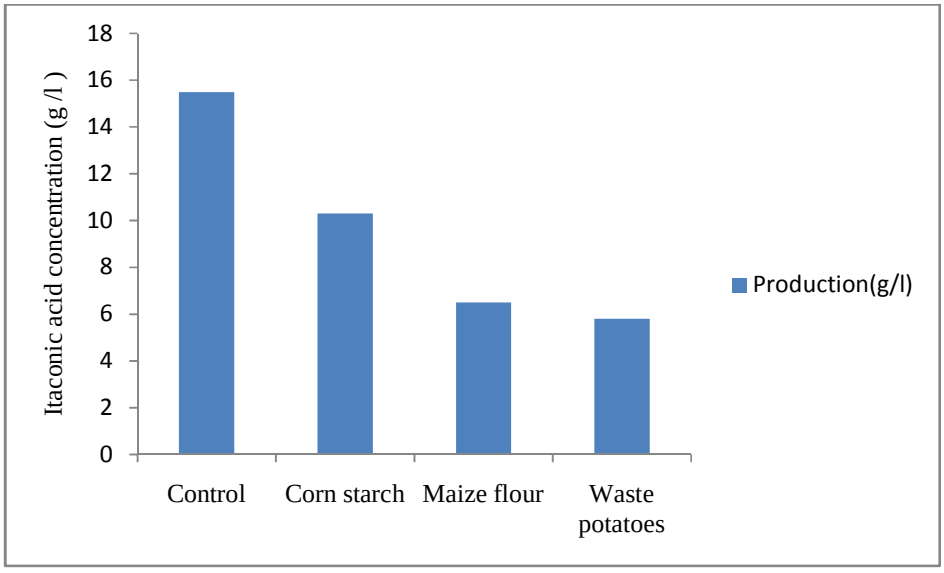

Fig. 3: Comparison of Itaconic acid production

Purification of itaconic acid by solvent extraction method

Purification of itaconic acid from broth was done by using n-Butanol as an extractant with the help of separating funnel. Effect of volume ratio between organic and the aqueous phase was investigated to get the maximum purification from broth. Initial itaconic acid concentration and organic-to aqueous volume ratio appears to have positive effect on the degree of extraction (Table 5).

Table 5. Degree of itaconic acid extraction as a function of organic to aqueous volume ratio

Substrate	Initial phase Volume(ml)		Equilibrium phase Volume(ml)		Equlibrium Concentration(M)		Degree of Extraction
	aqueous	butanol	aqueous	butanol	aqueous	butanol	
Corn Starch	10	10	9.5	10.5	3.25	24.37	74.28
	10	20	8	22	2.5	27.62	85.49
	10	**30**	**7**	**33**	**1.96**	**35.75**	**91.95**
	10	40	5	45	3.1	29.25	90.64
Maize flour	10	10	7	13	1.1	15.6	37.96
	10	20	5	25	0.6	16.9	40.3
	10	**30**	**3**	**37**	**0.1**	**22.75**	**42.05**
	10	40	2	48	0.9	21.12	41.08
Waste Potatoes	10	10	6	14	0.6	11.05	31.55
	10	20	5	25	0.2	14.3	33.06
	10	**30**	**4**	**36**	**0**	**17.55**	**33.64**
	10	40	3	47	0.2	15.92	33.29
Control	10	10	9	18	12.1	30.13	72.85
	10	20	8	22	11.7	35.01	95.17
	10	**30**	**8**	**32**	**11.5**	**40.8**	**97.65**
	10	40	7.8	42.2	12.5	38.4	89.12

Bromination method was performed for aqueous phase as well as organic phase to find out the concentration of itaconic acid after purification. Degree of itaconic acid extraction was found to increase significantly when the higher volume ratio between n-Butanol and starting aqueous solution was used in the process. At aqueous to organic ratio of 1:3, degree of extraction was highest and after that degree of extraction decreased. Consequently, extraction of itaconic acid with n- Butanol should be carried out with properly selected organic-to-aqueous volume ratio.

Itaconic acid purification

For control, itaconic acid concentration after purification was 40.8g/l. While for corn starch, itaconic acid concentration was found to be 35.75 g/l after purification. Likewise in case of maize flour, concentration was 22.75 g/l and for waste potatoes concentration of itaconic acid was 17.55g/l.

It was observed that purification by solvent extraction was found to be very successful method for purification because as shown in Fig. 4, After purification by solvent extraction method, concentration of itaconic acid was almost two or three times of the concentration after production for every raw materials.

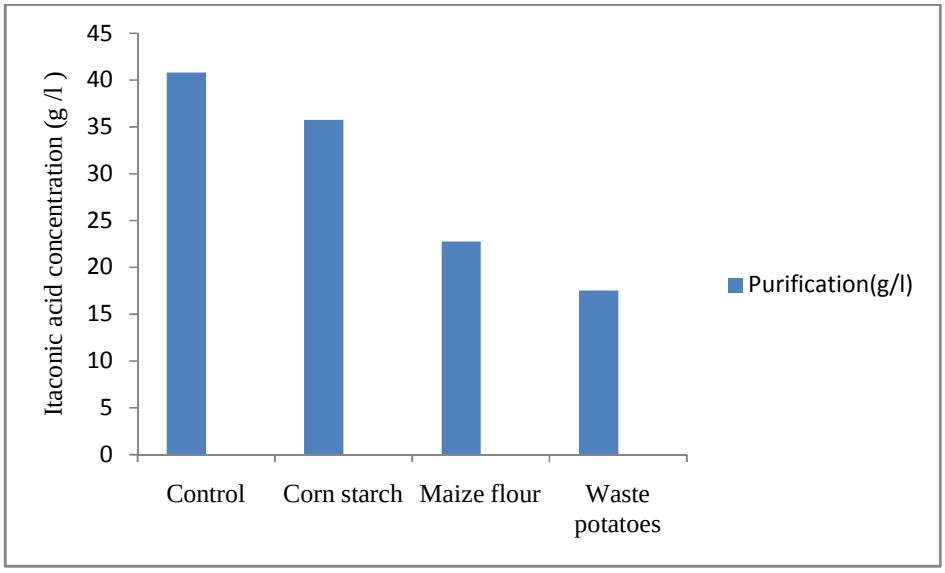

Fig. 4: Comparison of Itaconic acid purification

CONCLUSION

An efficient and low cost process can be established by production of itaconic acid utilizing cheap raw materials that can be helpful in improving the process economics more effectively while used at pilot scale. Similarly other cheap materials can also be used. Itaconic acid concentration was increased up to 2-3 times after purification by solvent extraction method which is inexpensive method for purification and also can be used for large scale operations. Other conditions like pH and temperature can also be optimized to increase the feasibility of the method.

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