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Comparative Studies on Downstream Processing and Fermentative Production of Itaconic Acid Using Aspergillus terreus

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In this study, a systematic process optimization was performed with an Aspergillus terreus MTCC 479. In the present study, cheap raw materials like Maize flour, Waste potatoes and Corn starch were used. Acid 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 120hr. After purification by Solvent extraction method by 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 cheap raw materials and also the use of inexpensive method for purification which will be helpful in decreasing the process economics.Keywords: Aspergillus terreus, Itaconic acid, Aspergillus oryzae, Bromination, Solvent extraction method.

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INTRODUCTION

Itaconic acid (2-methylidenebutanedioic acid) is an unsaturated di-carboxylic acid. It has a broad application spectrum in the industrial production of resins and is also used as a building block for acrylic plastics, acrylate latexes, super-absorbents, and anti-scaling agents [1-3]. The Itaconic acid derived Bis-pyrrolidone-type monomers are also used in preparation of Biopolymers [4]. Since the 1960s the production of itaconic acid is achieved by the fermentation with Aspergillus terreus on sugar containing media [1]. Although also other microorganisms like Ustilago zeae [5], U. maydis, Candida sp. [6], Rhodotorula sp. [7] Aspergillus flavus [21] were found to produce itaconic acid, A. terreus is still the dominant production host for production of Itaconic acid. [2; 8].The studies on biosynthesis of Itaconic acid reveals that it is derived via one of the intermediate of Tri carboxylic Acid (TCA) Cycle; cis aconitic Acid. During Biosynthesis the cis aconitic acid is converted to Itaconic acid via decarboxylation reaction performed through enzyme cis aconitate decarboxylase. Recent studies indicates that Macrophages as a immune response, expresses high levels of immunoresponsive gene 1 (IRG1) under inflammatory conditions as an enzyme that catalyses the production of Itaconic acid by decarboxylation of the Krebs cycle intermediate cis-aconitate.[9]. The optimal conditions for Itaconic acid clearly differ from conditions optimal for citric- and oxalic acid production [10]. The highest IA yield is achieved when glucose is used as the substrate, but crystalline glucose is too expensive to use as a raw material for the commercial production of IA. Therefore, other raw materials that are cheaper than crystalline glucose, such as starch [19], molasses, hydrolysates of corn syrup or wood, and other combinations, were also tested. The most frequently used substrates are beet or sugarcane molasses [11], which are pretreated by ion exchange or ferrocyanide [12] and increases the process economics. The present study demonstrates the production of itaconic acid utilizing Maize flour, the Corn starch and waste potatoes which are much cheaper than glucose helps in controlling the process economics. For purification, Solvent Extraction method is used which is inexpensive method than other methods like Liquid chromatograph. Also it is easy to scale up and permits continuous steady state operation.

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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 locally, Iso 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 (gl^{-1}) of Sucrose,30 ;Yeast extract,5; K_2HPO_4,1; $NaNO_3$,300; $MgSO_4.7H_2O$,50; KCl,50; $FeSO_4.7H_2O$,1; Agar15 .Aspergillus oryzae was propagated on Potato dextrose Agar (Hi-Media) with gl-1of PDA 24, pH-5.1.Slants were grown at 30^0 C for 5 days and stored at 4^0C

Acid Hydrolysis

Starch estimation in raw materials was done by anthrone method [13] and determination of reducing sugars was done by DNS method [14].Hydrolysis was performed by acid as well as by amylase enzyme produced from Aspergillus oryzae[17]. 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 [17, 18]. 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(g/l);Corn starch,24g; Yeast extract,36g; Na_2HPO_4,47g; KCl,0.2g; $MgCl_2$,0.2g; $CaCl_2$,1g.

Effect of incubation period

Effect of incubation period on amylase production was studied by measuring enzyme activity after every 24 hrs. Culture filtrate was harvested and enzyme assays was performed up to 120hrs. 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%) as well as time period (4, 8, 12, 16, 20, 24 hrs.) at 500C was studied to get maximum hydrolysis of substrates. After maximum hydrolysis, Centrifugation was done at 10000 rpm for 30 minutes 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% soluble starch (Merck) solution, 0.25 mL of 0.1 M 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 a high spore concentration. After incubation on rotary shaker at 200 rev/min for 24 hours at 350C, fractions of 10 ml were used to inoculate 90ml sterile production medium (l^{-1}) Glucose,100g; Ammonium sulphate, 2.36g; KH_2PO_4,0.11g; $MgSO_4.7H_2O$,2.1g; $CaCl_2.2H_2O$,0.13g; NaCl,0.074mg; $CuSO_4.5H_2O$,0.2mg; $FeSO_4.7H_2O$,5.5mg; $MnCl_2.4H_2O$,0.7mg; $ZnSO_4.7H_2O$,1.3 mg in 500 ml conical flasks. Cultures were then incubated for 6 days under the same conditions as above. Samples were taken after every 12 hrs till 6 days, diluted with deionized water to solubilize the itaconic acid and filtered through 0.2 µm whatmann discs .This sample was analysed for itaconic acid production by Bromination method [15].

Itaconic acid purification

Itaconic acid was purified by Solvent extraction method [20] by 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 [16].

RESULT AND DISCUSSION

Overall 95% starch content in Corn starch, 75% starch content in maize flour and 16% starch content was determined in waste potatoes by anthrone method. The starch content was found to be less in waste potatoes as compared to maize flour and corn starch. This may be due to more amounts of dietry fibres, fat content as well as more moisture content .Waste potatoes may also be 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%. Yield of reducing sugars 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 affective way. The table 1 showed that yield of reducing as well as hydrolysis percentage varied with substrate concentration. It was observed that for maize flour maximum yield of reducing sugars was 60 and hydrolysis was 80% with 10% substrate .For Corn starch maximum yield of reducing sugars 42.5 and 57% hydrolysis for 10% substrate was observed. While in case of waste potatoes, maximum yield of reducing sugars 17.5 and 73% hydrolysis for 15 % substrate was observed.

Effect of acid concentration on acid hydrolysis

Acid concentration was also varied in the ratio 1:0.5 to 1:5 for the same substrate concentration in which we got the maximum hydrolysis % (Table 1). Yield of reducing sugars and hydrolysis %(Table2) was calculated by using standard graph of Glucose. It was observed that acid concentration has significant effect on yield of reducing sugars as well as hydrolysis percentage. Table 1 shows effect of acid concentration on yield of reducing sugars as well as percentage hydrolysis. It was observed that for 10% maize flour, maximum yield of reducing sugars was 60 and hydrolysis was 80% with substrate to acid ratio 1:1. For 10% Corn starch, maximum yield of reducing sugars 42 and 56% hydrolysis with 1:1 substrate to acid ratio was observed. Likewise in case of 15% waste potatoes, maximum yield of reducing sugars 17 and 72% hydrolysis with 1:1 substrate to acid ratio was observed. In case of all the starch materials, significant effect up to a certain concentration of acid (1:1) 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. This might be due to the fact that high concentration of acid caused the reducing sugar degradation. The soft hydrolysis conditions led to a sugar-rich prehydrolysate. When using harsh pretreatment conditions, sugar recovery in raw materials decreased.

Effect of Incubation period on amylase production

The effect of incubation time for production of enzyme was also observed by varying the time period for production of amylase from 1st to 6th days. Enzyme activity was maximum on 4th day which was found to be 126 U/ml. (data not shown) The enzyme activity first increased with increasing time period i.e.it increased up to 4th day and then start decreasing till 6th day.The incubation period is directly related with the production of enzyme and other metabolic process up to a certain extent. Then after, production of enzyme started decreasing which might be due to the depletion of nutrients in

the medium which stressed the fungal physiology resulting in the inactivation of secretary machinery of the enzyme.

Optimization conditions for hydrolysis by amylase

After the production, the amylase enzyme was extracted from fermentation broth by centrifugation and used for further hydrolysis of maize flour, Corn starch and waste potatoes (Figure 2).The glucose produced by this hydrolysis method was further used for production of itaconic acid.

Figure 1 shows that for all three substrates, hydrolysis percentage was highest at 20th hour for 10% substrate. Maximum hydrolysis was found to be in case of maize flour i.e. 96%.In corn starch hydrolysis was found to be 67% while in case of waste potatoes hydrolysis was found to be 94% . For every substrate yield of reducing sugars and hydrolysis increased up to 20th hour then it became stable. This may be due to the reason that 20th hour time period was sufficient for all the substrate to get hydrolyzed. Further no substrate was left to be converted into glucose so 20th hour time period is optimized for hydrolysis of starch into glucose.10% substrate was found to be optimized to get the maximum hydrolysis for all the three starchy materials.Stable glucose production after 10% substrate might be 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 as shown in hydrolysis progress in Figure 1.

Comparison of Itaconic acid Production

The glucose released after hydrolysis of three 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. Pure glucose was used in control instead of glucose released after hydrolysis, to compare the production of itaconic acid. Samples were taken after every 24 hours and analysis of itaconic acid was done by Bromination method. Table 3 shows that maximum production of itaconic acid was at 120 hours for all three materials. For control, maximum production of itaconic acid was 15.5 g/l. 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. Production of itaconic acid was increasing with time period from 24 hours to 120 hours for all raw materials.

Maximum production of itaconic acid 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 may be due to amylolytic activity shown by A.terreus. As corn starch got completely liquefied after autoclaving of medium, so A.terreus shown more amylolytic activity in case of liquefied corn starch and hydrolyzed rest of starch also. So production of itaconic acid was more in corn starch.

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 4). Bromination method was performed for aqueous phase as well as organic phase to find out the concentration of itaconic acid after purification. Degree of extraction was calculated by using formulae. 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.

Comparison of Itaconic acid production and purification for all substrates

As shown in Table 4, 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 Figure 2, 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.

CONCLUSION

An efficient and low cost process can be established by production of itaconic acid utilizing cheap raw materials that can be helpful in decreasing the process economics more efficiently while used at pilot scale. Similarly other cheap materilas 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.

Figure 1: Optimization of time period and substrate concentration for hydrolysis by amylase

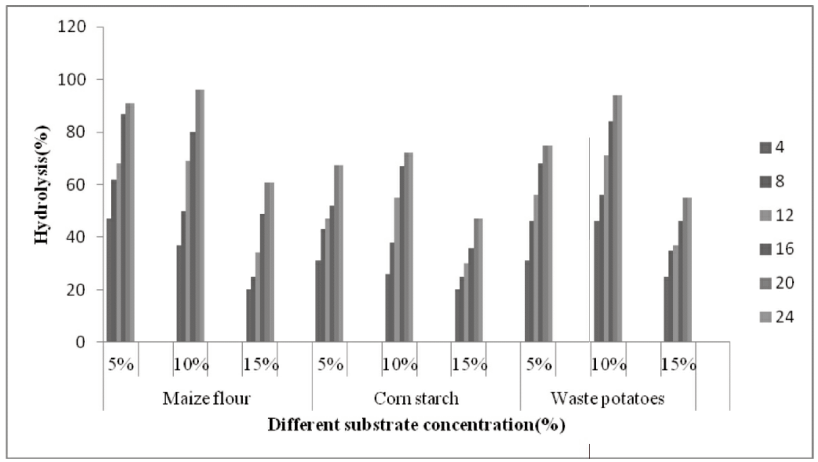

Figure 2: Comparison of Itaconic acid production and purification

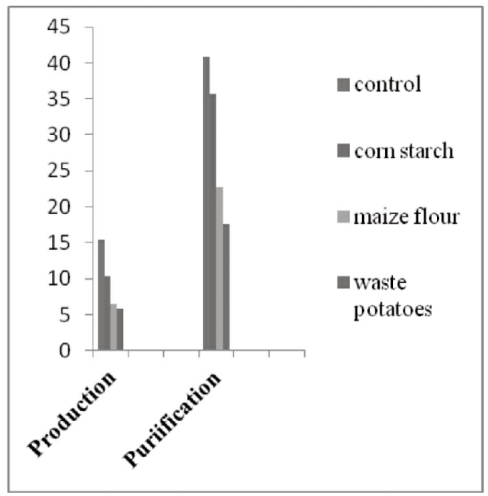

Figure 3: Optimization of time period and substrate concentration for hydrolysis by amylase

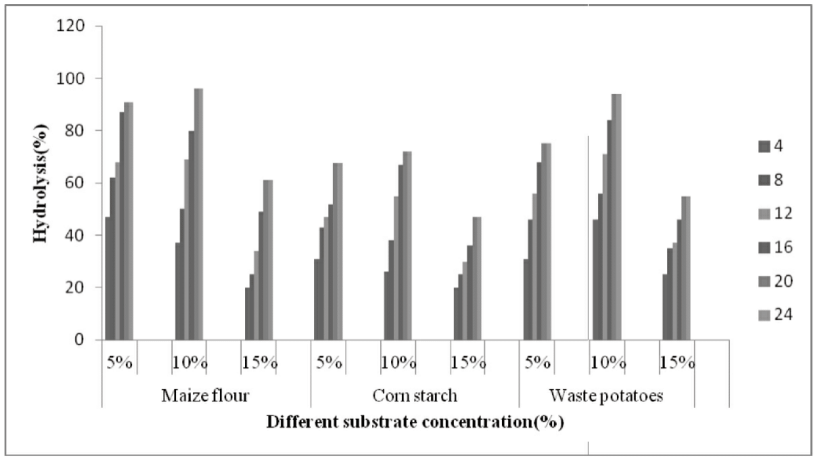

Figure 4: Comparison of Itaconic acid production and purification

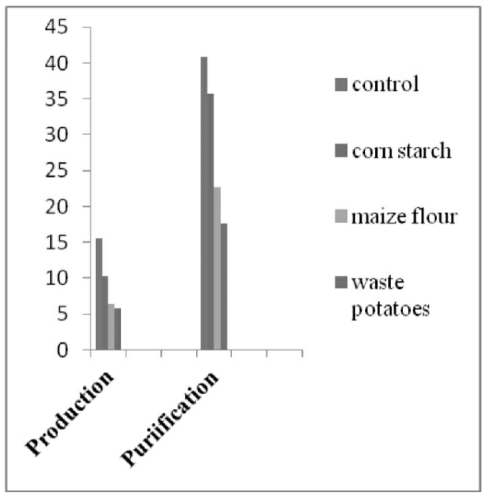

Table1: Effect of substrate concentration on yield of reducing sugars as well as Hydrolysis (%)

Substrate	Substrate concentration (%)	Absorbance (540nm)	yield of reducing sugars	Hydrolysis (%)
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

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	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

Table 3 Comparison of Itaconic acid production as estimated by Bromination method

Time(hour)	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

Table 4: 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

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