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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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Itaconic acid (IA) is a promising organic acid with a wide range of applications and the potential to open up new application fields in the area of polymer chemistry, pharmacy, and agriculture. In this study, a systematic process optimization was performed with an *Aspergillus terreus* MTCC 479 strain. In the present study, cheapest 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 126 U/ml) using *Aspergillus oryzae* NCIM 645. Itaconic acid production was 15.5 g/l from control (with pure glucose), 10.3 g/l from corn starch, 6.5 g/l from maize flour and 5.8 g/l from waste potatoes at 120 h. After purification by Solvent extraction method by using n-Butanol as solvent , Itaconic acid concentration was increased 2-3 times i.e. 40.80 g/l for control, 35.75 g/l for corn starch, 22.75 g/l for maize flour and 17.55 g/l for waste potatoes respectively using 1:3 aqueous to organic phase ratio. Therefore our present research is a comparative study for production of itaconic acid by cheap raw materials. This research also indicates the use of inexpensive method for purification contributing in reduction of the process economics.

Key words: Itaconic acid, maize flour, waste potatoes, corn starch, *Aspegillus terreus* MTCC 497, solvent extraction.

INTRODUCTION

Itaconic acid (2-methylidenebutanedioic acid, IA) is an unsaturated di-carbonic acid. It has a broad application spectrum in the industrial production of resins and is used as a building block for acrylic plastics, acrylate latexes, super-absorbents, and anti-scaling agents (Willke and Vorlop, 2001; Okabe *et al.*, 2009). Since the 1960s the production of itaconic acid is achieved by the fermentation with *Aspergillus terreus* on sugar containing media (Willke and Vorlop, 2001). Although also other microorganisms like *Ustilago zeae* (Haskins *et al.*, 1955), *U. maydis*, *Candida* sp. (Tabuchi *et al.,* 1981), and *Rhodotorula* sp. are found to produce itaconic acid, *A. terreus* is still the dominant production host for production of itaconic acid.(Okabe *et al.,* 2009; Kuenz *et al.*, 2012). 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, molasses, hydrolysates of corn syrup or wood, and other combinations, are also tested. The most frequently used substrates are beet or sugarcane molasses (Nubel and Ratajak, 1964), which are pretreated by ion exchange or ferrocyanide

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(Batti and Schweiger, 1963) and increase the process economics. The present research work demonstrates the production of itaconic acid utilizing maize flour, corn starch and waste potatoes which are cheaper materials than glucose and other materials and decrease the process economics. For purification, solvent extraction method is used which is inexpensive method than other methods like Ion exchange chromatography. Also it is easy to scale up and permits continuous steady state operation

MATERIALS AND METHODS

Strain and chemicals

Aspergillus terreus MTCC 479 and *Aspergillus oryzae* MTCC 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 locally. Isobutanol was purchased from Fischer scientific (Mumbai) and all other chemicals were of analytical reagent grade.

Culture media

Aspergillus terreus was grown on Czepak Dox medium which consists of (g/l) sucrose, 30 g ; yeast extract,5 g; K_2HPO_4 1 g; $NaNO_3$ 300 g; $MgSO_4 7H_2O$, 50 g; KCl, 50 g; $FeSO_4 . 7H_2O$,1 g; Agar 15 g. *Aspergillus oryzae* was propagated on Potato dextrose Agar (Hi-Media). 24 g/l, pH-5.1 inoculated slants were grown at 30 ^{0}C for 5 days and stored at 4 ^{0}C.

Acid hydrolysis

Starch estimation in raw materials was done by anthrone method (Morris 1948) and determination of reducing sugars was done by DNS method(Miller 1959).Hydrolysis was performed by acid as well as by 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, 5 ml 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^{0}C under shaking conditions for 24 h. This inoculum was further used for production of amylase enzyme in amylase production medium (g/l); corn starch, 24 g; yeast extract, 36 g, Na_2HPO_4, 47g; KCl, 0.2 g; $MgCl_2$,0.2 g; $CaCl_2$ 1 g.

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 assays was performed up to 120 h. After maximum production, whole broth was centrifuged at 15000 rpm for 30 minutes for extraction of enzyme.

Optimization of hydrolysis conditions

Effect of substrate concentration (5%, 10%,15%) as well as time period (4,8,12,16,20,and 24 h) at 50 ^{0}C 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 .One unit of amylase is defined as the amount of enzyme releasing 1 μ mol of glucose equivalent/ min under the assay conditions. Enzyme activity(U/ ml)=Concentration obtained from standard graph$\times$ Dilution factor $\times$1000/Time for enzyme incubation$\times$ 1g mole of substrate

Culture conditions for Itaconic acid production

Conidiospores from 7 day old culture slants were suspended in 5 ml sterile 0.05 mol/l phosphate buffer (pH 6.5) containing 0.1% Tween-80 and used to inoculate 500 ml conical flasks containing 100 ml sterile Czepak Dox medium to give a high spore concentration. After incubation on rotary shaker 200 rev/min for 24h. at 35^0C, fractions of 10 ml were used to inoculate 90ml sterile production medium (g/l) (Glucose,100 g; ammonium sulphate, 2.36 g; KH_2PO_4,0.11; $MgSO_4.7H_2O$, 2.1 g; $CaCl_2$. $2H_2O$, 0.13 g; NaCl,0.074 mg; $CuSO_4$. $5H_2O$,0.2 mg; $FeSO_4.7H_2O$, 5.5 mg; $MnCl_2$. $4H_2O$, 0.7 mg; $ZnSO_4.7H_2O$,1.3 mg) in 500 ml conical flasks. Cultures were then incubated for 6 days under the same conditions as above. Samples were taken every 12 h. till 6 days, diluted with deionized water to solubilize the itaconic acid and filtered through 0.2 µm whatman discs .This sample was analysed for itaconic acid production by Bromination method (Morris, 1945).

Itaconic acid purification

Itaconic acid was purified by solvent extraction method 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 (0.2 µm) 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 (500 ml) and allowed to settle for 1 h .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 phase were analyzed for determination of itaconic acid concentration by titration method in different ratios of n-butanol. Degree of extraction (%E) was calculated (Kanungnit Chawong, and Panarat Rattanaphanee, 2011).

RESULTS AND DISCUSSION

The starch content in corn starch, in maize flour and in waste potatoes was determined by anthrone method and which was found to be 95%, 75% and 16% respectively. 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 dietary fibers, 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 got decreased

Table 1 : Effect of substrate concentration on yield of reducing sugars as well as hydrolysis (%)

Substrates	Substrate concentration (%)	Yield of reducing sugar	Hydrolysis (%)
Maize flour	5	21.5	58
	10	**60**	**80**
	15	55	50
	20	52.5	35
Corn starch	5	20	47
	10	**42.5**	**57**
	15	32.5	30
	20	30	20
Waste potatoes	5	5	62
	10	11	68
	15	**17.5**	**73**
	20	15.5	49

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.

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 mg/ml 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

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

Substrates	HCl concentration (S:A Ratio)	Yield of reducing sugar	Hydrolysis (%)
Maize flour	1:0.5	42	56
	1:1	**60**	**80**
	1:3	55	73
	1:5	47.5	63
Corn starch	1:0.5	34	35
	1:1	**42**	**56**
	1:3	38	40
	1:5	35	37
Waste potatoes	1:0.5	12.5	52
	1:1	**17**	**72**
	1:3	15	63
	1:5	13	53

Table 3 : Comparison of Itaconic acid production

Substrates	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

we got the maximum hydrolysis percentage (shown in Table 1) Yield of reducing sugars and hydrolysis % (Table 2) 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 2 showed the 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 substrate to acid ratio of 1:1 was observed. Likewise in case of 15% waste potatoes, maximum yield of reducing sugars 17 and 72% hydrolysis with substrate to acid ratio 1:1 was observed.

Significant effect up to a certain concentration of acid (1:1) i.e. when acid concentration equivalent to the substrate concentration was observed in case of all the starch materials. 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 might cause the reducing sugar degradation. The soft hydrolysis conditions led to a sugar-rich prehydrolysate. When using

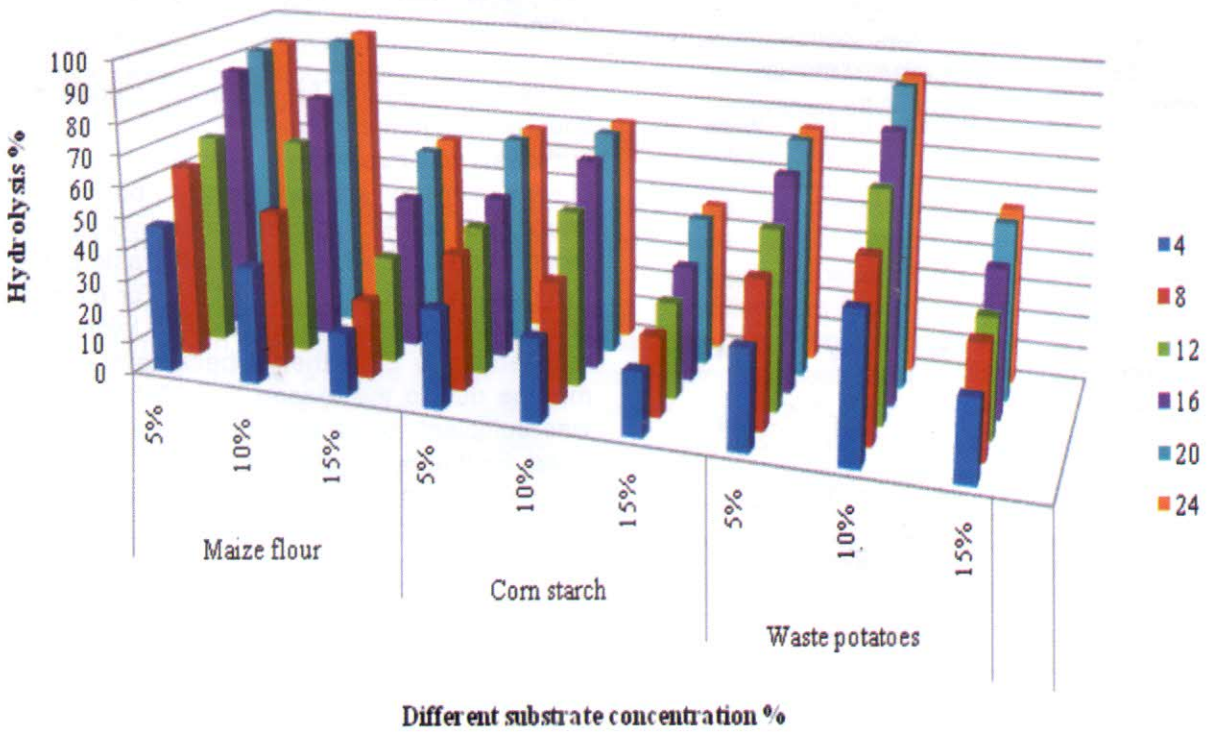

Fig. 1 : Optimization of time period and substrate concentration for hydrolysis percentage by amylase

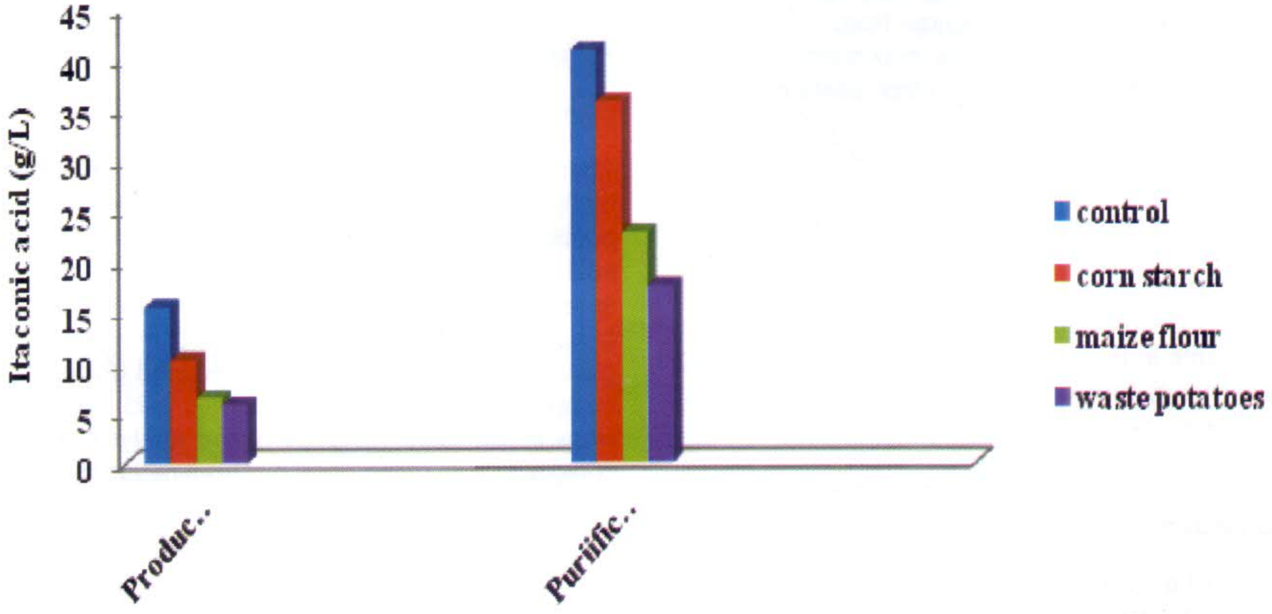

Fig. 2 : Compaison of production and purification

Table 4 : Degree of itaconic acid extraction as a function of organic to aqueous volume ratio

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

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 day 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 enzymes.

Optimization of conditions for hydrolysis by amylase

After the production, the amylase enzyme was extracted from fermentation broth by centrifugation at 15000 rpm for 30 minutes and was used for further hydrolysis of maize flour, corn starch and waste potatoes (Fig. 1).The glucose produced by this method was further used for production of itaconic acid.

Figure 1 showed that for all three materials maize flour, corn starch and waste potatoes, percentage hydrolysis was maximum at 20 h 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 20 h then it became stable. This may be due to the reason that 20 h time period was sufficient for all the substrate to get hydrolyzed. Further no substrate was left to be converted into glucose so 20 h time period is optimized for hydrolysis of starch into glucose. 10% substrate was found to be optimized as all the substrates were giving the maximum hydrolysis for this substrate concentration only.

Hydrolysis(%) was determined with varying concentration of substrate 5%, 10%, 15% and varying time periods of 4,8,12,16, 20, 24 h. It was observed that stable glucose production after 10% mostly due to enzyme inhibition by the present of impurities. Moreover, high concentration of substrate might have reduced the water content in reaction mixture which lowered pentose yield and also can lower the rate of hydrolysis as shown in hydrolysis progress in Fig 1. Low substrate concentration can cause in an increase of the glucose yield and hydrolysis percentage

Comparison of Itaconic acid production

After hydrolysis by amylase, the glucose produced by hydrolysis of maize flour ,corn starch and waste potatoes 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.Control was also kept in which pure glucose was used instead of produced glucose to compare the production of itaconic acid. Samples were taken after every 24 h and analysis of itaconic acid was being done by Bromination method. Production of itaconic acid(g/l) was calculated by using formulae.

Table 3 showed that maximum production of itaconic acid was at 120 h for every raw materials. In control maximum production of itaconic acid was

15.5 g/l. In maize flour production of itaconic acid was 6.5 g/l while while in case of waste potatoes and corn starch production was 5.8 g/l and 10.3 g/l respectively.Production of itaconic acid was increased with time period from 24 h to 120 h for all raw materials.

Maximum production of itaconic acid was in control in which pure glucose was used as raw material. With respect to all other raw materials, maximum production was found to be in case of corn starch i.e.10.3 g/l which may be due to amylolytic activity shown by *A.terreus*. As corn starch gets completely liquefied after autoclaving of medium due to which *A.terreus* amylolytic activity was more 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 the organic and the aqueous phase were investigated to get the maximum purification from broth. Initial itaconic acid concentration and organic-to aqueous volume ratio appeared 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 while in case of maize flour and waste potatoes, impurities were also present and also they were not liquefied during preparation of medium for production of itaconic acid. So amylolytic activity of *A.terreus* was not that much significant in these raw materials. Because of that production was less as compare to corn starch.

Comparison of Itaconic acid production and purification for all substrates

As shown in Table 4, for control, itaconic acid concentration after purification was 40.8 g/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 17.55 g/l.

It was observed that purification by liquid-liquid extraction was found to be very successful method for purification because as shown in Fig. 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.

Effect of the volume ratio between n-butanol and starting aqueous solution on degree of itaconic acid extraction was also significant. 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 maximum and after that degree of extraction decreased. Consequently, extraction of lactic acid with n-butanol should be carried out with properly selected organic-to-aqueous volume ratio.

Conclusion

An efficient and low cost process can be established by production of itaconic acid utilizing cheap raw materials that can helpful in decreasing the process economics, when 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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