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Extraction and quantification of acrylic acid from acrylamidase-catalyzed reaction produced by *Bacillus tequilensis*

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ABSTRACT

The reduction of non-renewable material has raised significant concerns for several years over the sustainable production of organic acids through bio-based methods in the world. One such way to overcome this problem is to use reactive extractants, in which appropriate extractants are employed to recover various organic and inorganic acids. The extraction of acrylic acid by solvent extraction is an illustration of this technique. The current study focuses on the synthesis of acrylic acid from acrylamidase produced by *Bacillus tequilensis*, succeeded by acid extraction from the amidase-catalyzed reaction by a solvent technique. Among the various solvents, ethyl acetate (2:1, v/v) was established as the most appropriate solvent for the extraction. Exactly 65 mg of raw acrylic acid was recovered from 20 ml of the amidase-catalyzed reaction. Various analytical methods such as thin layer chromatography, fourier transform infrared spectroscopy, high-performance liquid chromatography (HPLC), and mass spectrophotometry were accomplished for the identification, validation, and quantification of the extracted acrylic acid. The *m/z* value of acrylic acid obtained in the extracted product was 73.18, which was similar to the standard acrylic acid. From HPLC, almost 34% of bioconversion was quantified (3.4 mM) from 10 mM of acrylamide consumed. The extracted acrylic acid can be further exploited as chemical intermediates and pharmaceuticals in the future.

1. INTRODUCTION

Organic acids are essential industrial products in the world due to their multifaceted role. Their chemical production can be from non-renewable sources, such as fossils, or the biological fermentation of renewable biomass. The chemical pathway of production is mainly of concern these days due to intensification in the global warming of the environment. Therefore, there is a need to shift production pathways of organic acid toward green technologies [1,2]. The fundamental challenge of bio-based technology is the recovery of essential products from the downstream process in the form of synthesis broth or aqueous solution [3,4]. Reactive (liquid) extraction is considered the real prospect among different procedures for downstream processing. It provides primary conditions such as energy-saving, less time-consumption, higher yield, and less waste as byproducts in the separation of acids [5–7].

Acrylic acid or propenoic acid is a simple unsaturated carboxylic acid of the vinyl group with an R-carbon and a carboxylic acid terminal. It is a clean, monochrome liquid in pure form with a distinctive odor [8]. The production of acrylic acid is 4.2 million metric tons annually, positioned at 25 in the organic products list [9]. The market value of acrylic acid in 2013 was nearly 11 billion USD which is estimated to reach 9 million tons by 2025. The primary consumption of acrylic acid is in the synthesis of dispersants, polymeric flocculants, paints, coatings, and binders for paper, leather, adhesives, textile, and other commercial products in the industry [10–12].

Currently, industrial acrylic acid is manufactured through a two-step oxidation process from propylene through acrolein as an intermediate [4]. Firstly, propylene is oxidized to acrolein

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at 300°C–370°C; one–two bars using bismuth molybdate as a catalyst in the existence of steam and air. The synthesized yields are then subsequently served into the other reactor at 260°C–300°C using Molybdenum Vanadium Oxide substances as catalysts to synthesize the acrylic acid. From this process, more than 95% of the product is synthesized and the selectivity observed is nearly 85%–90%. These two-step procedures function primarily in the USA, Japan, England, and France. Another industrial production method of acrylic acid was the incomplete oxidation of propene – a one-step process in which yield was only 50%–60%. Meanwhile, propylene is obtained mainly from a non-renewable source (fossil), so the thrust for renewable sources is needed in the coming future [13] by a one-step process. Among the biomass-derivable materials, lactic acid, glycerol, and 3-hydroxy propionic acid have been reported for usage as simple ingredients for the manufacture of acrylic acid [14,15].

Through biotechnology, the production of organic acids has appeared to be little cost and energy effective, and environment friendly with few downstream challenges. The separation of the product (acid) may occur through fermentation processes that utilize renewable resources that are eco-friendly. But the major drawback is that significantly fewer products are separated through this challenging process, which is also expensive. Hence, an alternative method of acid separation, i.e., reactive extraction, is needed to meet the current requirement in the industry [8,16–18].

Reactive extraction is a hopeful process for obtaining the corresponding acid; however, it has diluent toxicity and is an active extractant [2,5,19]. Therefore, there is a prerequisite for utilizing less toxic extractants or diluents or a mixture of both in terms of non-toxicity that extract carboxylic acid competently. Different solvents, namely toluene, acetone, benzene, dichloromethane, ethyl acetate, paraffin, and sunflower oil, have been employed to obtain different acids from aqueous environments [20]. The extractant used for extraction binds reversibly with the broth's organic acid, which offers higher efficiency and selectivity of acids over others (non-acids).

The present study is the first to employ the extraction of a thermostable acrylamidase from *Bacillus tequilensis* (BITNR004; accession number MH978905) by several solvents, which was isolated from the hot spring of Surajkund in Ranchi, Jharkhand. The extracted acrylic acid (product) was studied by several analytical approaches like thin layer chromatography (TLC), fourier transform infrared spectroscopy (FTIR), high-performance liquid chromatography (HPLC), and mass spectrophotometry (MS) to identify, validate, and quantify the recovered acrylic acid.

2. MATERIALS AND METHODS

2.1. Chemicals

All the reagents and chemicals used during the study were procured from Himedia (Mumbai, India) and Sisco Research Laboratories, India and solvents were of HPLC grade (Merck, India). The glassware used throughout the study were from Tarsons, Borosil, and Rivera, India.

2.2. Microorganisms and culture conditions

Novel thermotolerant *B. tequilensis* bacteria were capable of synthesizing nitrile amide hydrolyzing enzyme. *Bacillus tequilensis* were isolated through the enrichment method employing mineral base media through acrylamide as an inducer. The sample was collected from a hot spring of Surajkund Jharkhand, India. *Bacillus* was conserved on Luria Bertani agar plates supplemented with 1 mM acrylamide as an inducer. On the contrary, amidase production was carried in shake flasks containing a mineral base medium with acrylamide as an inducer [21]. The bacterial cells were grown aseptically at 45°C for 24 hours under an agitation speed of 175 rpm in an orbital shaker.

2.3. Identification of isolated bacteria

The isolate was identified from the Microbial Type Culture Collection, Institute of Technology Chandigarh, India, and designated as *B. tequilensis*. The nucleotide sequence (16S rRNA) of the isolate *B. tequilensis* was submitted to the GenBank and was assigned an accession number MH978905.

2.4. Assay of amidase enzyme activity

The activity of amidase was carried out in 1.0 ml of the reaction mixture in the presence of specific substrates (final concentration = 10 mM), whole cells as biocatalyst (0.1 ml of whole cells adjusted to optical density as 2.0), and reaction buffer. The assay mixture was incubated at 50°C for 1 hour and the reaction was stopped by centrifugation at 10,000 rpm for 10 minutes. The ammonia liberated during the reaction was estimated from the supernatant and quantified at 640 nm by Berthelot's method. The activity of whole cell amidase (I.U.) is described as micromole of ammonia liberated from the enzyme substrate reaction per minute per ml at optimal conditions and calculated from the standard plot of ammonium chloride.

2.5. Extraction and quantification of acrylic acid

The acrylic acid synthesized in the enzyme-catalyzed reaction was extracted by a liquid–liquid extraction process using different solvents. The extracted product was analyzed by a number of procedures. Enzyme substrate reactions at different scales were prepared at operational conditions. Initially, 5 ml of the reaction mixture was analyzed, followed by extraction of acrylic acid [20]. Later on, the process was scaled up to 20 ml of the reaction mixture (enzyme assay) and pursued to extract acrylic acid at optimal conditions. The supernatant was collected after centrifugation and was acidified by hydrochloric acid (HCl) to pH 3.0. Then, various solvents like ethanol, diethyl ether, ethyl acetate, dichloromethane, hexane, benzene, toluene, acetone, and HC1 were added to the supernatant in double amounts. The liquid mixtures were firstly magnetically stirred at 600 rpm at room temperature (25°C ± 1°C) for 1 hour, left for 2 hours, and lastly dried in the rotary vacuum evaporator (Buchi Rotavapor R-205) [20]. After this, the residues obtained from the organic phase were subjected to various analytical methods like TLC, FTIR, HPLC, and MS to identify, validate, and quantify the recovered acrylic acid.

Table 1: Extraction and quantification of extracted acrylic acid with different solvents.

Extractant	Blank eppendorf tube weight (gm)	Eppendorf tube with extracted product weight (gm)	Extracted product (gm/20 ml)
Dichloromethane	1.02	1.04	0.02
Ethyl acetate	1.03	1.10	0.07
Benzene	1.03	1.04	0.01
Toulene	1.02	1.03	0.01
Hexane	1.03	1.03	0.00
Acetone	1.03	1.09	0.06
Isopropanol	1.03	1.05	0.02
Methanol	1.03	1.08	0.05

2.6. Identification, validation, and quantification of the extracted acrylic acid

2.6.1. Thin layer chromatography (TLC)

To investigate the extracted acrylic acid and the standard acrylic acid, TLC was accomplished on silica gel aluminum plate (Silica gel-60 F254, Merck). About 10 µl of both extracted samples and standard were spotted on the silica plate and retained in the developing chamber. The spots were developed in the mobile phase, consisting of methanol and water as 3.5:1.5 (v/v) and observed under UV light at 254 nm [21]. Finally, the identification of spots was confirmed against reference acid as R_f values.

2.6.2. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectrum of extracted acrylic acid and standard acid was recorded by an FTIR spectrophotometer (IRPrestige 2, Shimadzu Corporation, Japan). The analytes study was carried out by potassium bromide (KBr) cells and stated in the series of 500–4,000 cm^{-1} as wave number [22,23]. Different bands obtained from the FTIR spectra were analyzed from several sources.

2.6.3. High-performance liquid chromatography (HPLC)

The quantification of acrylic acid extracted through liquid extraction was carried out through HPLC (Waters, Miliford) equipped with C18 reverse phase column and photodiode array detector. The mobile phase used for elution of the product (acrylic acid) consisted of water and acetonitrile (90:10 v/v) as optimized under gradient elution with a flow rate of 1 ml/minute; 210 nm [21]. The extracted acid with the standard acrylic acid was analyzed to examine the percentage of biotransformation. The HPLC peak area for different acrylic acid concentrations (1–10 mM) was prepared against retention time (minute) to estimate the concentration of acid extracted (Fig. 3a and b).

2.6.4. Mass spectrophotometry

Similarly, molecular mass of the extracted acrylic acid and standard acrylic acid was studied by mass spectrophotometry (LC-MS; Thermoscientific Linear Trap Quadropole-XL). The MS investigation conditions were as follows: desolvation temperature 600°C; capillary voltage 0.4 kV; and cone and desolvation gas flow rate was 50 and 1,200 l/hour [20]. The *m*/*z* value of the extracted acid obtained was compared with the *m*/*z* value of the standard acrylic acid.

3. RESULT AND DISCUSSION

3.1. Extraction of acrylic acid

Thermostable amidase synthesized from *B. tequilensis* produced acrylic acid at a higher temperature and at a neutral pH. This study illustrates the importance of the synthesis of acrylic acid in an eco-friendly manner. The acrylic acid obtained after the biotransformation reaction using amidase enzyme synthesized by whole cells of *B. tequilensis* was extracted by various solvents like acetone, isopropanol, toluene, methanol, dichloromethane, hexane, ethyl acetate, and benzene (2:1; v/v) (Table 1). The extraction capability of the solvent is symbolized by the distribution

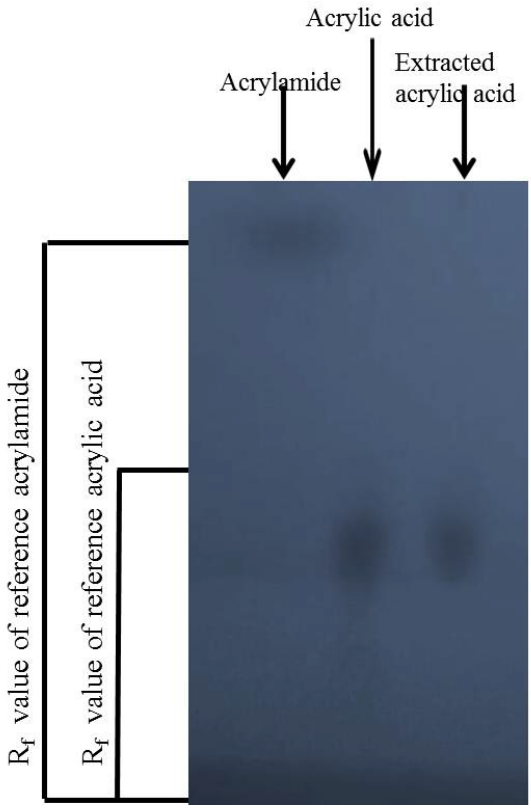

Figure 1: TLC of standard acrylamide (lane 1); standard acrylic acid (lane 2); and extracted acrylic acid (lane 3).

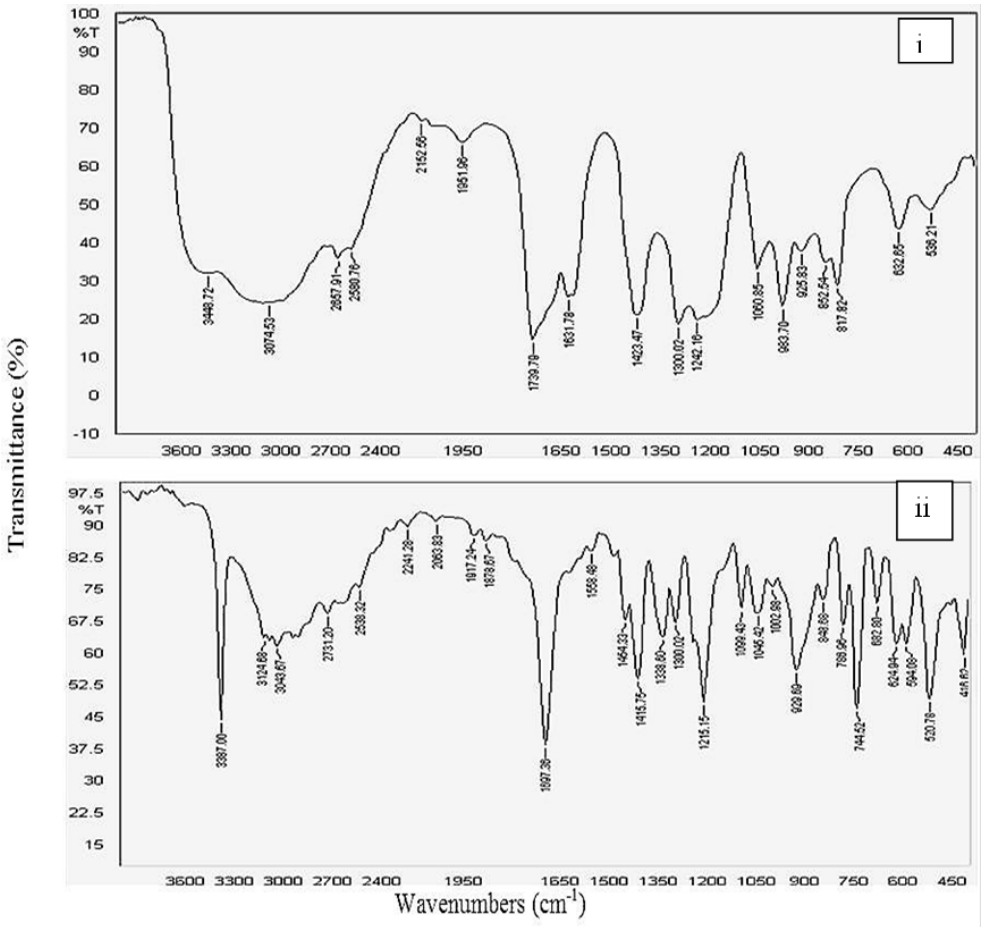

Figure 2: FTIR spectrum of (i) standard acrylic acid and (ii) extracted acrylic acid.

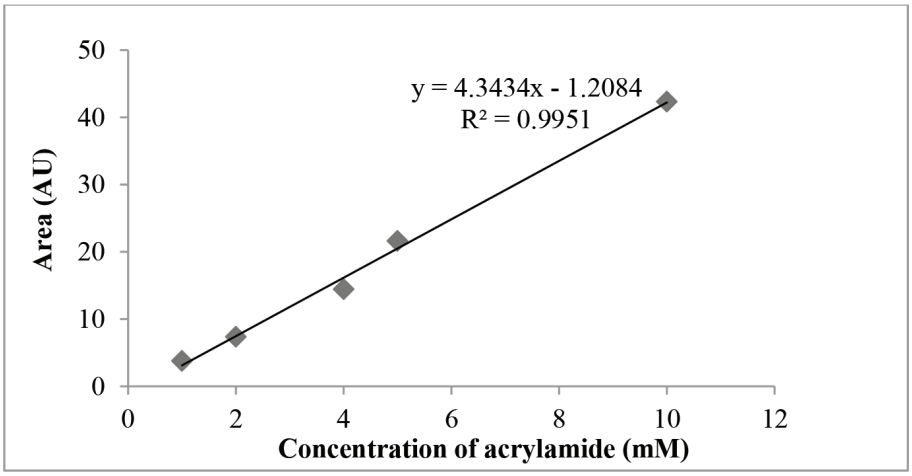

Figure 3a: Standard peak area plot of acrylamide.

coefficient (K_d), which is stated as the amount of acrylic acid present in the organic phase (Corg) to the amount of acid present in the aqueous phase (Caq),

$$K_d = \text{Corg/Caq}$$

The amounts of the extracted acrylic acid from different solvents are mentioned in Table 1. Among all the solvents, ethyl acetate yielded maximum product, followed by acetone and methanol. Approximately 65 mg crude acid was extracted using ethyl acetate as a solvent. The crude acid was further studied by TLC, FTIR,

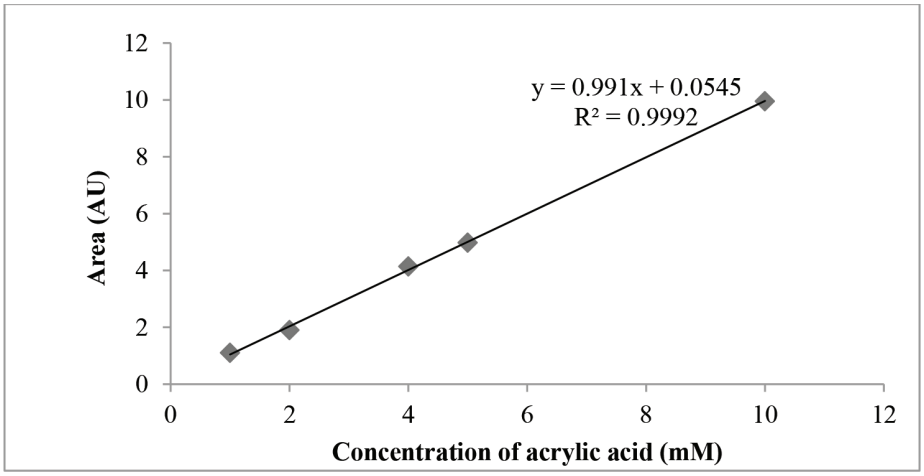

Figure 3b: Standard peak area plot of acrylic acid.

HPLC, and MS. The negligible amount of acrylic acid is present in the aqueous phase and hence it was not quantifiable. This result is similar to Tesnim and Kumar [20], where the distribution coefficient (K_d) of nicotinic acid was less during extraction.

3.2. Identification and validation of biocatalytic activity of extracted acrylic acid

3.2.1. Thin layer chromatography (TLC)

The extracted acrylic acid was analyzed by TLC along with standard acrylamide and acrylic acid, respectively. The samples were loaded on aluminum silica gel plates (Silica gel-60 F254, Merck), visualized under UV light at 254 nm. The R_f values of standard acrylamide (0.91) and acrylic acid (0.49) were matched with the extracted product (Fig. 1) which displayed a similar R_f value to acid (0.49) suggesting the extraction of amidase-catalyzed biotransformation of acrylamide to acrylic acid.

3.2.2. Fourier transformation infrared spectroscopy (FTIR)

The extracted acrylic acid along with the standard acrylic acid was analyzed for numerous functional groups as wave numbers by use of FTIR, as shown in Figure 2.

The hydrolysis of amides to acid was confirmed by the presence of a different functional group at different wave numbers by FTIR spectroscopy (Shimadzu corp. Japan, IR- prestige 2; KBr cells) [21,24]. The stretching and bending of carboxylic acid was observed in the region of 1,690–1,740 cm^{-1} wave number, while 1,697.36 cm^{-1} showed carboxylic acid bond formation in the reference sample. The extracted acrylic acid exhibited similar wave numbers as the functional groups compared to the standard acrylic acid, thus establishing extraction and recovery of acrylic acid from the enzyme-catalyzed reaction system.

3.2.3. High-performance liquid chromatography (HPLC)

The HPLC method was used to determine the identity and quantitative analysis of extracted acrylic acid. According to the chromatogram, the retention time of standard acrylic acid (1.826 minute) was linked to the extracted acrylic acid (1.826 minute) (Fig. 4). The standard peak area plot of various acrylic acid concentrations was used to investigate the quantification of acrylic acid recovered. Using 10 mM of acrylamide as a substrate, the extracted crude sample recovered almost 3.4 mM from the standard plot of acrylic acid (Fig. 3a and b). Different concentrations of inducers were used to find out the synthesis of intracellular amidase as expressed in terms of amidase activity. We demonstrate that under standard conditions, about 34% of acrylic acid was biotransformed into acrylamide via the acrylamidase enzyme. A study was conducted in 2010 to extract acrylic acid using isobutyl acetate (3:1) produced from amidase of *Trichosporon asahii* 2-1. Biotransformation efficiency was reported to be 89.5% from HPLC [25].

3.2.4. Mass spectrophotometry (MS)

The analysis of the molecular mass of extracted acrylic acid was conducted through mass spectrophotometry (LC-MS; Thermoscientific Linear Trap Quadropole-XL). The mode of ionization shown in Figure 4a is in the negative ion mode, while Figure 4b shows the positive ion mode. The extracted product displayed a peak at *m*/*z* value of 73.18, which resembles to the standard acrylic acid as $a \pm 1$ ionization value (Fig. 5a and b). Apart from acrylic acid, propyl acrylate was also observed at 113.49 *m*/*z* value.

4. CONCLUSION

The extraction of acrylic acid by solvent extraction draws more attention because of its good recovery and eco-friendly route. However, extraction efficiency might be improved by the reactive extraction method, where proper extractants are employed to recover acids. The growing demand for acrylic acid withdraws consideration toward the green scheme for synthesizing and extracting acrylic acid from amidase-catalyzed reactions. The present study is an effort of extracting acrylic acid through a solvent in an eco-friendly manner. The extracted sample analyzed by various analytical tools

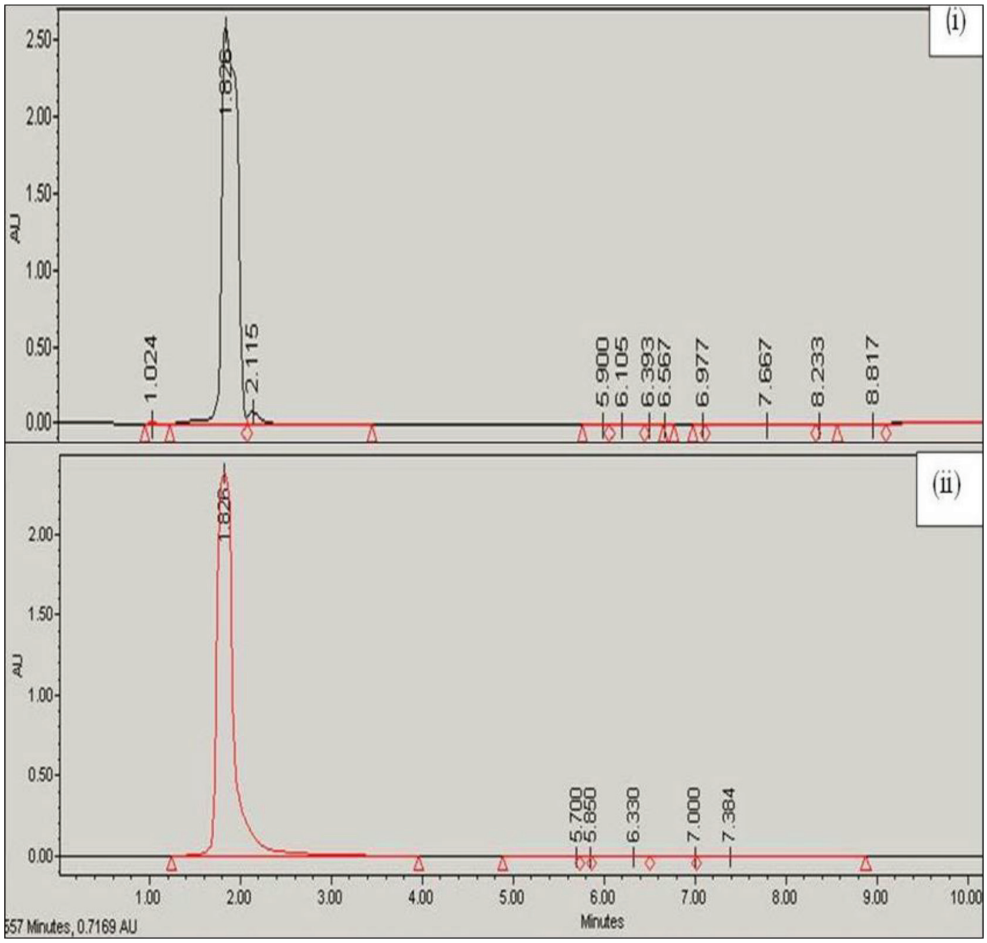

Figure 4: HPLC chromatogram of (i) standard acrylic acid and (ii) extracted acrylic acid catalyzed by acrylamidase.

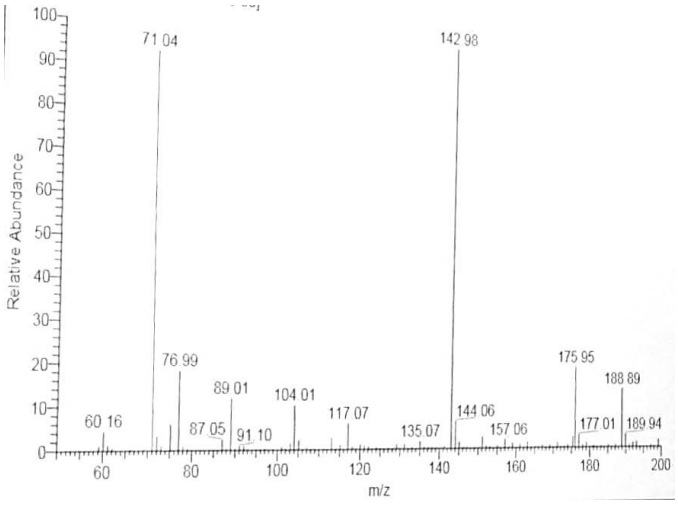

Figure 5a: MS of the standard acrylic acid.

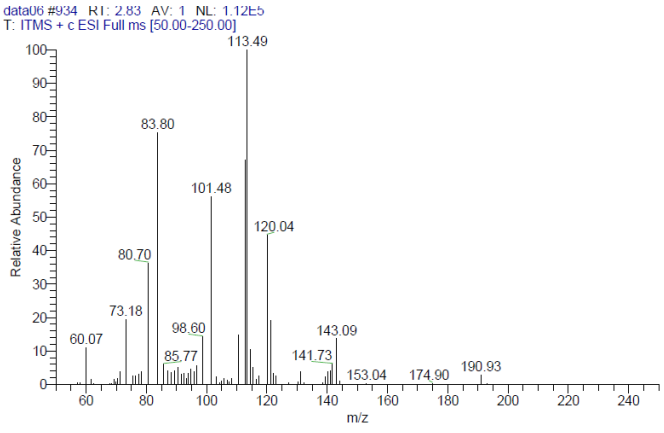

Figure 5a: MS of the extracted acrylic acid.

confirmed the presence of acrylic acid with a recovery of about 34%. Furthermore, the reactive extraction protocol serves as an efficient downstream processing method for various organic and inorganic acids.

5. ACKNOWLEDMENT

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6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

7. FUNDING

There is no funding to report.

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

REFERENCES

1. Reyhanitash E, Zaalberg B, Ijmker HM, Kersten SR, Schuur B. CO_2-enhanced extraction of acetic acid from fermented wastewater. Green Chem 2015;17(8):4393–400; doi: 10.1039/C5GC01061F
2. Zhou Z, Li Z, Qin W. Reactive extraction of saturated aliphatic dicarboxylic acids with trioctylamine in 1-octanol: equilibria, model, and correlation of apparent reactive equilibrium constants. Ind Eng Chem Res 2013;52(31):10795–801; doi: 10.1021/ie4008002
3. Straathof AJ, Sie S, Franco TT, Van der Wielen LA. Feasibility of acrylic acid production by fermentation. Appl Microbiol Biotechnol 2005;67(6):727–34; doi: 10.1007/s00253-005-1942-1
4. Xiaobo XU, Jianping L, Peilin C. Advances in the research and development of acrylic acid production from biomass. Chinese J Chem Eng 2006;14(4):419–27; doi: 10.1016/S1004-9541(06)60094-3
5. Kumar S, Pandey S, Wasewar KL, Ak N, Uslu H. Reactive extraction as an intensifying approach for the recovery of organic acids from aqueous solution: a comprehensive review on experimental and theoretical studies. J Chem Eng Data 2021;66(4):1557–73; doi: 10.1021/acs.jced.0c00405
6. Altunay N, Elik A, Gürkan R. Extraction and reliable determination of acrylamide from thermally processed foods using ionic liquid-based ultrasound-assisted selective microextraction combined with spectrophotometry. Food Addit Contam Part A 2018;35(2):222–32; doi: 10.1080/19440049.2017.1394585
7. Chen J, Zheng RC, Zheng YG, Shen YC. Microbial transformation of nitriles to high-value acids or amides. Biotechnol China I 2009; 113: 33–77. doi: 10.1007/10_2008_25
8. Tuyun AF, Uslu H. Reactive extraction of acrylic acid using trioctylamine (TOA) in versatile diluents. Desalin Water Treat 2015;55(1):193–8; doi: 10.1080/19443994.2014.911707
9. Kar A, Bagde A, Athankar KK, Wasewar KL, Shende DZ. Reactive extraction of acrylic acid with tri-n-butyl phosphate in natural oils. J Chem Technol Biotechnol 2017;92(11):2825–34; doi: 10.1002/jctb.5295
10. Bhalla TC, Kumar V, Kumar V, Thakur N. Nitrile metabolizing enzymes in biocatalysis and biotransformation. Appl Biochem Biotechnol 2018;185(4):925–46; doi: 10.1007/s12010-018-2705-7
11. Gong JS, Shi JS, Lu ZM, Li H, Zhou ZM, Xu ZH. Nitrile-converting enzymes as a tool to improve biocatalysis in organic synthesis: recent insights and promises. Crit Rev Biotechnol 2017;37(1):69–81; doi: 10.3109/07388551.2015.1120704
12. Wu Z, Liu C, Zhang Z, Zheng R, Zheng Y. Amidase as a versatile tool in amide-bond cleavage: From molecular features to biotechnological applications. Biotechnol Adv 2020;43:107574; doi: 10.1016/j.biotechadv.2020.107574
13. Lin MM. Selective oxidation of propane to acrylic acid with molecular oxygen. Appl Catal A Gen 2001;207(1-2):1–6; doi: 10.1016/S0926-860X(00)00609-8
14. Sun J, Yu H, Chen J, Luo H, Shen Z. Ammonium acrylate biomanufacturing by an engineered *Rhodococcus ruber* with nitrilase overexpression and double-knockout of nitrile hydratase and amidase. J Ind Microbiol Biotechnol 2016;43(12):1631–9; doi: 10.1007/s10295-016-1840-9
15. Gong JS, Lu ZM, Li H, Shi JS, Zhou ZM, Xu ZH. Nitrilases in nitrile biocatalysis: recent progress and forthcoming research. Microb Cell Fact 2012;11(1):1–8; doi: 10.1186/1475-2859-11-142
16. Wasewar KL. Reactive extraction: an intensifying approach for carboxylic acid separation. Int J Chem Eng Appl 2012;3(4):249; doi: 10.7763/IJCEA.2012.V3.195
17. Wasewar KL, Shende D, Keshav A. Reactive extraction of itaconic acid using tri-n-butyl phosphate and aliquat 336 in sunflower oil as a non-toxic diluent. J Chem Technol Biotechnol 2011;86(2):319–23; doi: 10.1002/jctb.2500
18. Keshav A, Wasewar KL, Chand S. Extraction of propionic acid with tri-n-octyl amine in different diluents. Sep Purif Technol 2008;63(1):179–83; doi: 10.1016/j.seppur.2008.04.012
19. Zhou X, Liu L, Chen Y, Xu S, Chen J. Efficient biodegradation of cyanide and ferrocyanide by Na-alginate beads immobilized with fungal cells of *Trichoderma koningii*. Can J Microbiol 2007;53(9):1033–7; doi: 10.1139/W07-070
20. Arfi T, Nigam VK. Extraction and quantification of nicotinic acid from 3-cyanopyrdinase catalysed reaction system. Res J Chem Environ 2018; 22 (9): 7–13.
21. Prabha R, Nigam VK. Biotransformation of acrylamide to acrylic acid carried through acrylamidase enzyme synthesized from whole cells of *Bacillus tequilensis* (BITNR004). Biocatal Biotransfor 2020;38(6):445–56; doi: 10.1080/10242422.2020.1780211
22. Pacheco R, Karmali A, Serralheiro ML, Haris PI. Application of fourier transform infrared spectroscopy for monitoring hydrolysis and synthesis reactions catalyzed by a recombinant amidase. Anal Biochem 2005;346(1):49–58; doi: 10.1016/j.ab.2005.07.027
23. Pacheco R, Serralheiro ML, Karmali A, Haris PI. Measuring enzymatic activity of a recombinant amidase using fourier transform infrared spectroscopy. Anal Biochem 2003;322(2):208–14; doi: 10.1016/j.ab.2003.07.012
24. Kolev T, Spiteller M, Koleva B. Spectroscopic and structural elucidation of amino acid derivatives and small peptides: experimental and theoretical tools. Amino Acids 2010;38(1):45–50; doi: 10.1007/s00726-008-0220-9
25. Rubin-Bejerano IF, Fink GR, Osler H. Canadian patents database/patent 2760044 summary. Canadian Intellectual Property Office, Gatineau, Canada, 2010.

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