academicJournals

Vol. 13(10), pp. 1182-1187, 5 March, 2014
DOI: 10.5897/AJB2013.13326
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Fumaric acid production by *Rhizopus oryzae* and its facilitated extraction via organic liquid membrane

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Accepted 29 January, 2014

Microbial fermentations are efficient alternatives to different manufacturing practices being followed for industrially important compounds like organic acids, food and pharmaceutically active products. However, the attractions offered by microbial processes are overshadowed by the costs associated with downstream processing involved in the recovery of pure products from such systems. Reduction of the cost in terms of financial and energy related inputs have been the goal of recent ongoing research in the field of bio-processing. This study has been focused towards development of an efficient method for simultaneous extraction of products from microbial fermentation. Through the study, the domain of liquid membranes and the possibilities of energy efficient extraction of microbial products from ongoing processes have been investigated. Liquid membrane system involves a liquid which is immiscible with the source and receiving solutions serves as a semi permeable barrier between these two liquid phases. The study uses a fumaric acid produced through fermentation of *Rhizopus oryzae* on Potato Dextrose Broth (PDB) under aerobic conditions as a model sample for extraction analysis. The culture media, that is presumably rich in fumaric acid and some other acidic products, has been subjected to extraction through liquid membrane based process. The reduction in concentration of sample used as source is measured titrimetrically. Thin layered chromatography has been deployed at certain instances for qualitative verification of the concentrations. The set up consisted of a modified layered 'liquid membrane' setup for a 'fumaric acid' source, with toluene as organic membrane and sodium hydroxide as strip phase. The liquid membrane contained a carrier for assisted transfer and was agitated. Maximum extraction takes place during the first 20 to 30 min of the run as fumaric acid concentration falls to almost 40% of its initial concentration.

Key words: Fumaric acid, carrier, trioctylamine (TOA), liquid membrane, facilitated extraction.

INTRODUCTION

Polymeric and inorganic membranes are used commercially for many applications including gas separations (Tabe-Mohammadi, 1999; Irabien et al., 2013), water purification (Zhang et al., 2008), food and diary-(Decloux et al., 2001), petrochemical (Karguri et al., 2009), pharmaceutical- (Lindner et al., 2001; Ceynowa and Koter, 2003) and biotechnology industry (Zydney and Reis, 2001; Charcosset, 2006; Escudero et al., 2013). The international nomenclature and definition of the membrane and membrane separation has been well described (Strathmann, 2006; Ulbricht, 2006). If membranes are viewed as semi permeable phase separators, then the traditional concept of membranes as polymer films can be extended to include liquids. They are defined as Liquid Membranes (LMs). Liquid membrane system involves a liquid which is immiscible with the source(feed) and receiving (strip) solutions that serves as a semi permeable barrier between these two

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liquid and gas phases. The term liquid membrane transport includes processes incorporating liquid-liquid extraction and membrane separation in one continuously operating device. It utilizes an extracting reagent solution, immiscible with water, stagnant or flowing between two aqueous solutions (or gases), the source or feed and receiving or strip phases. In most cases, the source and receiving phases are aqueous and the membrane organic, but the reverse configuration can also be used (Katalin and Petra). Liquid membranes possess higher selectivity values than solid membranes and further increase in mass flux and selectivity have been reported by incorporating some carrier, which reacts reversibly and selectively with a specific permeate in the liquid membrane (Kocherginsky et al., 2007; Ravanchi et al., 2010; Kaur and Vohra, 2010).

Research and development activities within these disciplines involve diverse applications of liquid membrane technology, such as gas separations (Baker, 2002; Golemme et al., 2009), recovery of valued or toxic metals (Alizadeh et al., 2002; Kulkarni et al., 2002; Othman et al., 2006, 2004; Manchanda et al., 2011; Janardan et al., 2012), removal of organic compounds and recovery of fermentation products and some other biological systems (Schlosser et al., 2005; Dimitrov et al., 2005; Heerema et al., 2006; Boyadzhiev at al., 2006). In the last decade, studies have been carried out towards the application possibilities of ionic liquids in liquid membrane processes for the transport and separation of solids, liquids and gases (Endres et al., 2008; Koel, 2008; Wasserchied, 2007). A new class of liquid membrane called micro-encapsulated liquid membrane has also been investigated for the production of oxygen enriched air (Figoli et al., 2001). Microbial productions are being followed for industrially important compounds like proteins, organic acids, food and coloration agents and pharmaceutically active products. Organic acids production by filamentous fungi and their associated metabolic pathways have also been reviewed (Goldberg et al., 2006; Magnuson and Lasure, 2004). Fumaric acid is a naturally occurring four-carbon dicarboxylic acid that is finding increasing use as a food acidulant and beverage ingredient.

Fumaric acid has many potential industrial applications, ranging from the manufacture of synthetic resins and biodegradable polymers to the production of intermediates for chemical syntheses (Roa et al., 2008). Recently, three strains of fungus *Rhizopus* oryzae were screened to produce fumaric acid using untreated and treated corn distillers' dried grains (Thomas, 2008). Microbial productions have even been discussed using bacterial strain, *Lactobacillus* (Donnelly et al., 2001). However, the attractions offered by microbial processes are overshadowed by the costs associated with downstream processing involved in the extraction of pure products from such systems. As such, recovery techniques in submerged cultivation for fumaric acid have been scarcely studied in comparison to organic acids citric acid (Heinzle et al., 2006) and lactic acid (Joglekar et al., 2006).

Downstream processing such as reactive extraction and membrane electrodialysis have not been studied whereas simultaneous fermentation and adsorption have been studied for the same (Joglekar et al., 2006). Reduction of the cost in terms of financial and energy related inputs has been the goal of our investigations. This study has been focused towards development of an efficient method for simultaneous extraction of products from microbial fermentation. Currently, fumaric acid is produced from petroleum based derivative maleic anhydride and as the petroleum prices are rising quickly, the cost of fumaric acid production has also increased. The study uses a fumaric acid produced through fermentation of *R*. oryzae using potato dextrose broth (PDB) under aerobic conditions as a model sample for extraction analysis. The culture media, that is presumably rich in fumaric acid and some other acidic products, is subjected to extraction through liquid membrane based process. The non-miscible phases are arranged in a manner which is known as bulk liquid membrane (BLM) (Belafi-Bako et al., 2000; Clark et al., 2005). The reduction in concentration of sample used as source is measured titrimetrically.

Thin layered chromatography was deployed at certain instances for qualitative verification of the concentrations detected or undetectable through titrimetric analysis. Certainly, improvements are desirable for up-scaling the proposed method.

MATERIALS AND METHODS

Membrane solutions were prepared by dissolution of trioctylamine (TOA) (Fluka A.G. Switzerland 95%) as an extractant in toluene (S.D. Fine chemicals, 99.5%). Fumaric acid (LOBA Chemicals) solutions dissolved in water were used for controls. Strip phase solutions were prepared by dissolving sodium hydroxide pellets (Qualigens, Glaxo 97.7%) in water. Phenolphthalein indicator, methyl orange and hydrochloric acid (Qulaigens) were used for titrations. Chloroform and methanol (E. Merck 99%) were used for thin layer chromatography analysis. The lyophilized stocks *R. oryzae* were procured from MTCC, IMTECH Chandigarh. The strain is attributed with high levels of fumaric acid production. Potato dextrose agar (Himedia) and potato dextrose broth (Himedia) were used to maintain the culture strain. The media was inoculated with a lyophilized culture of a strain of *R. oryzae* MTCC-262 and incubated at 30°C for 7 days to allow fungal growth. After seven days, mycelia growth was observed on media's surface. Subculturing was carried out in 50 ml media using hyphal spores.

Set-up

The basic setup to perform experimental studies was created out of borosilicate glass molded as per the desired specifications. The assembly consisted of two concentric cylinders, the inner being shorter in height to accommodate for the liquid membrane that would connect the two liquids (Figure 1). The similar experimental apparatus has also been reported by us for the extraction of

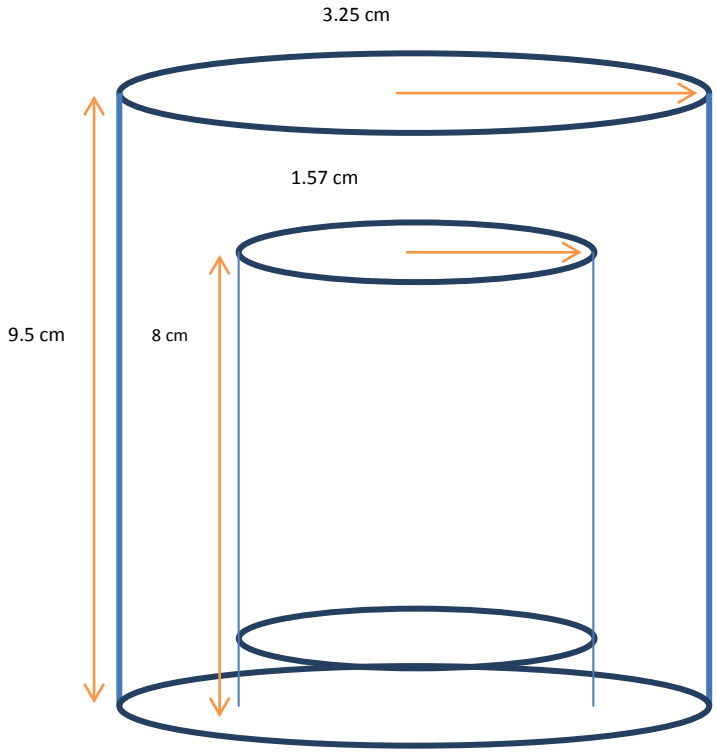

Figure 1. Specifications of the setup for holding the three liquids.

propionic and acetic acids. The facilitated extraction for the same has also been described in the same paper (Kaur and Vohra, 2010).

Source feed

The source contains components of interest that need to be recovered. In this study, samples obtained from the fermentation of *R. oryzae* were used as source feed for investigations. Pure fumaric acid solutions were used as controls in the experiments. The concentration of pure samples used was optimally kept in the ppm range to mimic actual fermentation concentrations in order to optimize the setup for such applications.

Strip phase

Under normal circumstances, strip phase for any separation process being carried out by a liquid membrane has high affinity for the product of interest yet easily dissociates from it under the effect of slight variation in conditions being provided or on the action of a chemical reagent. Keeping in mind these two characteristics, the best possible option that came up for the strip phase was an alkaline medium. Sodium hydroxide has high affinity for organic acids leading to the formation of sodium salts and water.

Liquid membrane

The liquid membrane used should be completely immiscible in the source and strip phase and should be able to maintain a distinct interface that supports high rates of diffusion across it. Given that the two other liquids selected exist in stable aqueous solutions (polar in nature) so a non polar liquid membrane was a clear choice. Toluene, with its high immiscibility in aqueous phases and easy availability, was the liquid membrane of choice.

Carrier

The carrier molecule is the essence of the whole setup – it efficiently binds to the product of interest and then releases it on coming in contact with the strip phase. The ease with which it attaches to and releases the product is of major interest and the selection of such a molecule was necessary that could fulfill both the requirements for organic acids and also be insoluble in aqueous solvents. These requirements were fulfilled to the fullest by trioctylamine (TOA) which is used as an extractant for organic acids in industrial processes and is immiscible in aqueous solutions.

Transport mechanism theory

The reaction equilibrium for the fumaric acid in the present set-up can be defined by taking into account two interfaces I and II defined as feed-phase:membrane phase interface and strip-phase:membrane phase interface, respectively. Complex formation of the extractant TOA with the fumaric acid is proposed in the mechanism at interface I.

$mFA\ (aq) + nTOA\ (org) \rightarrow FA_m.TOA_n\ (org)$

The acid-amine complex diffuses from interface I to interface II across the membrane where the extractant is released.

Table 1. Variation of fumaric acid concentration and percentage extraction during reactive extraction process in fermentation samples of different days of fermentation.

Sample time (min)	Amount of fumaric acid /100 ml							
	Day 1		Day 2		Day 5		Day 6	
	Amount of FA/100 ml	Percentage extraction	Amount of FA/100 ml	Percentage extraction	Amount of FA/100 ml	Percentage extraction	Amount of FA/100 ml	Percentage extraction
0	0.1323	-	0.1822	-	0.3017	-	0.2855	-
10	0.0943	28.73	0.1184	35.02	0.1950	35.36	0.1706	40.24
20	0.0856	35.30	0.0987	45.83	0.1695	45.82	0.1486	47.95
30	0.0885	33.10	0.0789	56.70	0.1184	60.75	0.1172	58.94
40	0.0783	40.82	0.0789	56.70	0.1137	62.32	0.1137	60.18
50	0.0783	40.82	0.0789	56.70	0.1126	62.68	0.1114	60.98
60	0.0812	38.62	0.0777	57.35	0.1126	62.68	0.1102	61.40

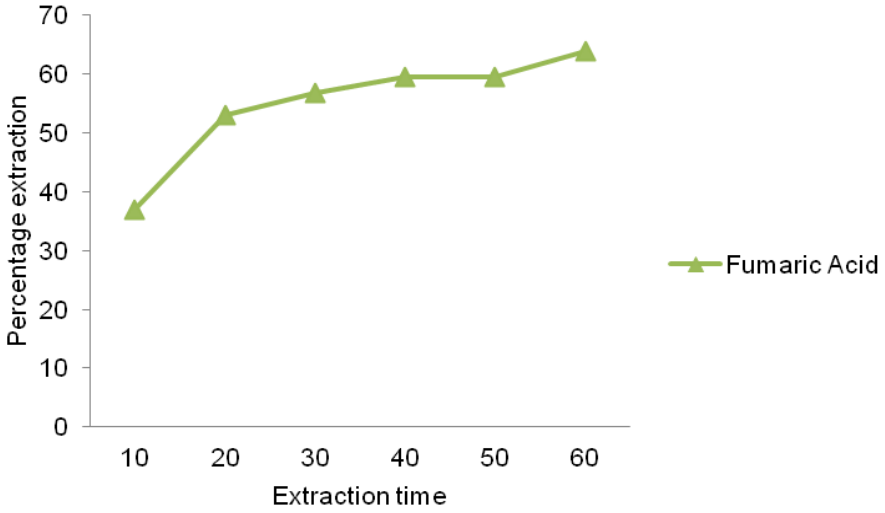

Figure 2. Trend of percentage extraction of fumaric acid (1 g/100 ml) in the proposed BLM set-up.

$FA_m.TOA_n$ (org) → FA (org) + TOA (org)

TOA diffuses back to the interface I across the membrane. The ionized acid reacts further with the strip phase comprised of sodium ions and produces a non-diffusive compound. Transfer mechanisms for the recovery of acetic acid, propionic acid and fumaric acid have been described elsewhere in a similar fashion (Kaur and Vohra, 2010; Zhang et al., 2009).

RESULTS

Experiments to investigate the extraction potential of the proposed BLM set-up was carried out firstly with pure fumaric acid (1 g/100 ml) where above 60% extraction has been achieved (Figure 2). Secondly, cell-free supernatant was then used as source feed to investigate the extraction potential of the proposed BLM for fumaric acid from a mixed solution. It was observed that fumaric acid concentration increases as fermentation proceeds. It was also observed that the concentration of fumaric acid falls with time for all the samples when subject to reactive extraction process (Table 1). The concentration of fumaric acid drops significantly during first few minutes of experiment and then becomes almost constant. Investigations were carried out with cell-free supernatant as obtained from fermentation of *R. oryzae* on different days of fermentation as a source feed along with 1 N sodium hydroxide as strip phase and 4% (v/v) TOA in toluene as liquid membrane. As initial concentration of fumaric acid increases with fermentation days, maximum extraction achieved also increased from 38 to 61% (Figure 3). It is further concluded that the maximum percentage extraction for the two cases remained close to 60%. Based on the results, it is confirmed that the proposed BLM set-up has the potential to extract the fumaric acid both pure and from a mixture of other acids

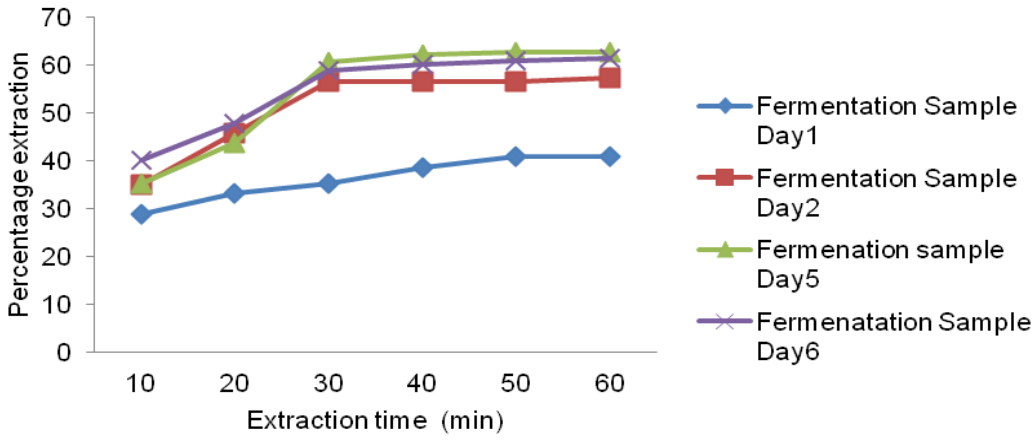

Figure 3. Variation of percentage extraction of fumaric acid in the samples obtained from fermentation of *Rhizopus oryzae*.

and that too even in dilute concentrations. Further studies are required to optimize the system and make the technique commercially viable so that it can be used for *in-situ* extraction of fumaric acid from the solution.

In a similar set of studies, recovery of fumaric acid from waste solutions have been reported using a different carrier triakylamine by hollow-fibre supported liquid membrane (Zhang et al., 2009). The study has investigated several different classes of solvents along with cosolvents. Combination of the reverse osmosis and complex extraction and stripping has also been reported for the treatment of industrial wastewater containing fumaric acid (Zhang et al., 2008). Therefore, the current study confirms that the proposed set-up has potential to recover fumaric acid from wastewater and fermentation broth and could emerge as a fast, simpler and cheaper alternate for the same.

Conclusion

Based on the literature survey, *R. oryzae* appeared to be the microorganism with the highest productivity of fumaric acid. The fumaric acid production can be improved by exploring the field of genetic engineering with the help of acid-resistant strain. Another alternative is to replace petroleum based maleic acid and to use fermentatively produced fumaric acid. This production can be enhanced by overcoming product inhibition by applying *in-situ* removal of fumaric acid during fermentation.

The latter has been achieved partly with the help of current studies. Further optimizations in terms of carrier, carrier concentration and strip phase concentration are thus desirable to make the technique commercially viable. The validation of our findings and improvements thereafter promise considerable reduction in the costs associated with processes deployed for production of different industrially important microbial metabolites. Apart from reduction of overall costs, the application of these principles can also enhance productivity in cases of self-inhibited processes through simultaneous removal of product.

REFERENCES

Alizadeh N, Salimi S, Jabbari A (2002). Transport study of palladium through a bulk liquid membrane using hexadecylpyridinium as carrier. Sep. Purif. Technol. 28:173-180.

Baker WR (2002). Future directions of membrane gas separation technology. Indus. Engg. Chem. Res. 41(6):1393-14112.

Belafi-Bako K, Gubicza L, Mulder M (2000). Integration of membrane processes into Bioconversions, KluwerAcademic Publishers ISBN: 978-3064-6437-9, New York.

Boyadzhiev L, Dimitrov K, Metcheva D (2006). Integration of solvent extraction and liquid membrane separation: an efficient tool for recovery of bio-active substances from botanicals. Chem. Eng. Sci. 61:4126-4128.

Ceynowa J, Koter I (2003). Kinetic resolution of chiral alcohols in bifunctional membrane exhibiting enzyme activity and enantioselective permeation. J. Mol. Catal. B: Enzymatic 24-25:17-26.

Charcosset C (2006). Membrane processes in biotechnology: an overview. Biotechnol. Adv. 24:482-492.

Clark JD, Han B, Bhown AS, Wickramasinghe SR (2005). Amino acid resolution using supported liquid membranes, Sep. Purif. Technol. 42(3):201-211, 13835866.

Decloux M, Daufin G, Escudier JP, Carrere H, Berot S, Fillaudeau L (2001). Recent and emerging applications of membrane processes in the food and dairy industry. Trans Institution of Chemical Engineers 79 (c). 89-102.

Dimitrov K, Metcheva D, Boyadzhiev L (2005). Integrated processes of extraction and liquid membrane isolation of atropine from *Atropa belladonna* roots. Sep. Purif. Technol. 46:41-45.

Donnelly M, Millard CS, Stols L (2001). Mutant E.coli strain with increased succinic acid production. USRE37393.

Endres F, Abbott AP, MacFarlane DR (2008). Electrodeposition from Ionic liquids. Wiley-VCH Verlag GmbH & Co. KGaA. ISBN: 978-3-527-31565-9, Weinheim, Germany.

Escudero I, Geanta RM, Ruiz MO (2013). Micellar-enhanced ultrafilteration for the recovery of lactic acid and citric acid from beet molasses with sodium dodecyl sulphate. J. Memb. Sc. 430: 11-23.

Figoli A, Sager WFC, Mulder MHV (2001). Facilitated oxygen transport in liquid membranes: review and new concepts. J. Memb. Sci. 181,

97-110.
Goldberg I, Rokem JS, Pines O (2006). Organic acids: old metabolites, new themes. J. Chem. Techol. Biotechnol. 81:1601-1611.
Golemme G, Bernardo P, Driolo E (2009). Membrane gas separation: A review/state of the art. Indus. Engg. Chem. Res. 48(10):4638-4663.
Heerema L, Roelands M, Hanemaaijer JH, Bont J de, Verdoes D (2006). In-situ phenol removal from fermentation broth by pertraction. Desalination 200:485-487.
Heinzle E, Biwer AP, Cooney CL (2006). Development of sustainable bioprocess-modelling and assessment. Wiley, Chichester.
Irabien A, santos E, Albo J, Daniel CI, Portugal CAM, Crespo JG (2013). Permeability modulation of supported magnetic ionic liquid membranes (SMILMs) by an external magnetic field. J. Memb. Sc. 430: 56-61.
Janardan P, Panja S, Mohapatra PK, Tripathi SC, Gandhi PM (2012). Supported liquid membrane transport studies on Am (III), Pu(IV), U(VI) and Sr(II) using irradiated TODGA. J Hazard Mater. 237-38:339-346.
Joglekar HG, Rahman I, Babu S, Kulkarni BD, Joshi A (2006). Comparative assessment of downstream processing options for lactic acid, Sep. Purif. Technol. 52, 1-17.
Karguri A, Ravanchi MT, Kaghazchi T (2009). Application of membrane separation processes in petrochemical industry: a review. Desalination 235:199-244.
Katalin BB, Petra C. Application of ionic liquids in membrane separation processes. www.intechopen.com.
Kaur A, Vohra DK (2010). Study of bulk liquid membrane as a separation technique to recover acetic and propionic acids from dilute solutions. Indian J. Chem. Tech. 17:133-138.
Kocherginsky NM, Yang Q, Seelam L. (2007). Recent advances in supported liquid membrane technology. Sep and Purif Technol. 53(2):171-177.
Koel M (2008). Ionic liquids in chemical analysis. CRC Press Taylor & Francis Group. ISBN: 978-1-4200-4646-5, USA.
Kulkarni PS, Mukhopadhyay S, Bellary MP, Ghosh SK (2002). Studies on membrane stability and recovery of uranium(VI) from aqueous solutions using a liquid emulsion membrane process. Hydrometallurgy 64:49-58.
Lindner W, Maier NM, Franco P (2001). Separation of enantiomers: needs, challenges, perspectives. J. Chromat. A 906:3-33.
Magnuson JK, Lasure LL (2004). Organic acid production by filamentous fungi. In: Tracz JS, Lange L(eds) Advances in fungal biotechnology for industry, agriculture and medicine. Kluwer/Plenum, New York, USA, 307-340.
Manchanda VK, Bhattacharyya A, Mohapatra PK, Gadly T, Raut DR, Ghosh SK (2011). Liquid-liquid extraction and flat sheet supported liquid membrane studies on Am(III) and Eu(III) separation using 2,6-bis(5,6-dipropyl-1,2,4-triazin-3-yl)pyridine as the extractant. J. Hazard Mater. 195:238-244.
Othman N, Mat H, Goto M (2006). Separation of silver from photographic wastes by emulsion liquid membrane system. J. Memb Sci. 282, 171-177.
Othman N, Mat H, Goto M (2004). Liquid membrane technology for precious metals recovery from industrial waste. http://eprints.utm.my/1064/1/NorasikinOthman2004_LiquidMembrane TechnologyFor Precious.pdf
Ravanchi MT, Kaghazchi T, Kargari A (2010). Supported liquid membrane separation of propylene-propane mixtures using a metal-ion carrier. Desalination 250(1):130-135.
Roa Engel CA, Straathof AJ, Zijlmans TW, van Gulik WM, van der Wielen LA (2008). Fumaric acid production by fermentation. Appl. Microbiol. Biotechnol. 78(3), 379-389.
Schlosser S, Kertesz R, Martak J (2005). Recovery and separation of organic acids by membrane-based solvent extraction and pertraction: an overview with a case study on recovery of MCPA. Sep. Purif. Technol. 41:237-266.
Strathmann H (2006). Application of membrane processes for the separation of molecular mixtures. European J. Lipid Sci. Technol. 82(4):152-166.
Tabe-Mohammadi A (1999). A review of the applications of membrane separation technology in natural gas treatment. Sep. Sci. Technol. 34(10):2095-2111.
Thomas PW (2008). Fumaric acid production by Rhizopus oryzae on corn Distillers' Grains with solubles. Res. J. Microbiol. 3(1), 35-40.
Ulbricht M (2006). "Advanced functional polymer membranes" (Invited Feature Article), Polymer 47, 2217-2262.
Wasserchied P, Welton T (2007). Ionic liquids in synthesis. VCH-Wiley ISBN:978-3-527-31239-9, Weinheim, Germany.
Zhang L, Li S, Zhang J, Zhi T, Chen H (2008). Combination of complex extraction with reverse osmosis for the treatment of fumaric acid industrial waste water. Desalination 234:362-369.
Zhang L, Li SJ, Chen HL (2009). Recovery of fumaric acid by hollow-fiber supporting liquid membrane with strip dispersion using trialkylamine carrier. Sep. Purif. Technol. 66:25-34.
Zydney A, Reis RY (2001). Membrane separations in biotechnology. Curr. Opinion Biotechnol. 12:208-211.