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Lactic acid production from free and Polyurethane immobilized cells of *Rhizopus oryzae* MTCC 8784 by direct hydrolysis of starch and agro-industrial waste

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Abstract

This study investigated the production of L-lactic acid (LA) from agro-industrial waste by free and polyurethane immobilized *Rhizopus oryzae* MTCC 8784 using the innate hydrolytic potential of the fungus. Fungal biomass and lactic acid production obtained from the agrowastes (from all 3 pre-treatments) were compared to the yield parameters obtained with starch. Sugar bagasse on acid hydrolysis gave the highest lactic acid yield of 79.2 g/L, with highest volumetric productivity of 1.1 g/L/h. Cultivation of polyurethane (PU) sponge immobilized *R. oryzae* MTCC 8784 on starch utilization at 30°C, 160 rpm of agitation rate and pH of 6.0 for 72 h, showed 1.15 fold increase in LA concentration in comparison to free cells. Lactic acid was successfully obtained from amylolytically saccharified starch by 4 repeated batch cultures with stable efficiency. All the agrowaste substrates tested supported higher lactic acid yield as compared to free cells using PU immobilized *R.oryzae* MTCC 8784 at 30°C, 160 rpm of agitation and pH of 6.0 for 72 h with sugar bagasse showing a maximum yield of 88.2g/L (1.22 fold higher than with free cells). HPLC chromatogram revealed lactic acid peak with a retention time of 2.61 min. To our knowledge, this is the first report on lactic acid production by innate hydrolysis of agrowastes under immobilized condition by a fungus. The isolate MTCC 8784 can be a good candidate for cost-effective and ecofriendly industrial production of lactic acid from agro-industry waste.

Introduction

Lactic acid has a significant role as a bulk chemical in different industries such as food, leather, cosmetic, chemical etc. The most important field of contemporary lactic acid utilization is as a monomer for production of poly-lactides (PLA). Poly-lactides have many favorable characteristics like biodegradability, biocompatibility, elasticity and good controlled drug release profile. Fermentative production of lactic acid is a dominant industrial production route (Gao *et al.*, 2011). Traditional feedstocks for lactic acid production are starch based substrates which are also utilized for food production (Bilanovic *et al*., 2011). Utilization of renewable, inexpensive and abundant agro- industrial waste for the production of L- Lactic acid is an increasing trend in recent years. Van-Thuoc *et al*. (2007) reported that in order to use the agro-industrial residues as fermentation substrates, these should be subjected to hydrolysis step for the release of easily metabolizable sugars. Acid and enzymatic methods are the two main reported methods for hydrolysis, but acid hydrolysis requires more energy for heating and is relatively difficult to control. It also necessitates corrosion resistant materials since it gives rise to high colour and salt and ash content. An economical process to hydrolyze agrowastes using innate enzymes for lactic acid production by free and immobilized cells has scarcely been found.

The fungus *R. oryzae* is widely studied as a commercially perspective producer of L(+)-LA (Miura *et al.*, 2004), because the fungal cells possess better resistance to high concentrations of accumulated LA (Schepers *et al*., 2004) compared to the commonly used bacterial producers. Besides this advantage, the fungi can use media with much lower contents of nutrient components compared to those required by bacteria (Hujanen *et al.*, 2001). The use of *R. oryzae* in immobilised form is one of the most efficient approaches to improving the LA production process (Tay and Yang 2002). Immobilization makes separating the liquid medium from the cells much easier (Nedovic and Willaert, 2004) and facilitates multiple reuses of fungal cells for long-term LA production. Several researchers have attempted to use immobilization techniques for L(+)-lactic acid production with *R. oryzae*. The entrapment methods using soft gels such as Ca-alginate have mostly been employed in some studies (Hang *et al.*,

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1989). In gel-entrapping methods, the limitation of oxygen supply because of diffusional resistance might decrease the fermentation rate and/or L(+)-lactic acid transformation efficiency. The problems, associated with filamentous fungal fermentations can be overcome with cell immobilization on support polymer matrix (Dong *et al*., 1996).

In light of the above perspective, the present study attempts bioconversion of agro-industrial wastes in free and immobilized conditions for L-Lactic acid production by the innate enzymatic potential of *R.oryzae* MTCC 8784 alleviating the need of pretreatment of complex agrowastes thus reducing the production cost.

Materials and Methods

Microorganism, media and culture conditions

Lactic acid producing strain of *R. oryzae* MTCC 8784 (Microbial Type Culture Collection and Gene Bank, Chandigarh), was maintained on Sabouraud dextrose agar slants and stored at 4°C. After growth and sporulation, 10 ml of distilled water with wetting agent was aseptically added to each agar slants which were then scraped to release the spores. This spore suspension was centrifuged at 4000 rpm for 10 min; the spores were washed and resuspended in 1 ml distilled water. Then, 500 µl spore suspension containing $(6.8\pm1.2)\times10^7$ spores ml^{-1} was used to provide spore inoculum for each of 250 ml shake-flask containing 50 ml of the medium. The flasks were then incubated on a rotary shaker at 30°C and 160 rpm for 3 days, unless otherwise specified. The fermentation medium contained (g/L): starch 40, yeast extract 10, peptone 20, K_2HPO_4 0.5, KH_2PO_4 0.5, $MgSO_4.7H_2O$ 0.05, $(NH_4)_2SO_4$ 2, pH 6.0.

Substrates

Eight different agro-food wastes procured from the local markets of Bangalore, Karnataka, viz. rice bran, wheat bran, ragi bran, rice starch water, tea waste, sugar cane bagasse, groundnut and coconut oil cakes were chosen for Lactic acid fermentation. The substrates were oven dried at 60˚C, pulverised followed by pretreatments like steam explosion, direct hydrolysis and acid hydrolysis and used as carbon source for LA fermentation.

Pretreatment of agrowastes

The modified method of Pumiput *et al.* (2008) was used for substrate hydrolysate preparation. 100 g of each agro-food waste was steam-exploded in autoclave at 121°C for 20 min. Water was added to the wet pre-treated material to make up the volume to 1 L and boiled at 80°C for 30 min. The content of the flasks were cooled and volume made up to 1L with distilled water. Later the hemicellulose hydrolysate was recovered by filtration with cheese cloth. The steam exploded liquid hydrolysate thus obtained was supplemented with salts as mentioned above, autoclaved and used as media for LA production by *R.oryzae* MTCC 8784. Acid post hydrolysis of hemicellulose hydrolysate was carried out to cleave the xylooligosaccharides into monomeric sugars by autoclaving at 121°C with H_2SO_4 (2% v/v) for 30 min. Chemical pre-treatment is to remove chemical barriers, so the enzymes can have access to cellulose for microbial destruction. Post acid hydrolysis, the hemicellulose hydrolysate was recovered by filtration with cheese cloth. The liquid hydrolysate from acid post hydrolysis was treated with $Ca(OH)_2$ to adjust the pH to 6-6.8 and the $CaSO_4$ precipitates were removed by filtration with Whatmann filter paper No.1. The neutralized substrate thus obtained was supplemented with salts as mentioned above, autoclaved and used as media for LA production by R.oryzae MTCC 8784 (Pumiput *et al*., 2008). Direct infusion was carried out by drying and pulverizing the agro-wastes. The powdered substrates (100 g/L) were added as the sole carbon source and the media was supplemented with minimal salts, autoclaved and used for LA fermentation (Pumiput *et al.*, 2008).

Immobilization of R.oryzae *in polyurethane foam matrix*

Foam matrices (15 ppi; pore per inch) were used throughout the work. Prior to use, the support materials was submerged in distilled water and autoclaved three times for 15 min at 121°C, the distilled water was replaced each time to remove any chemical that might have otherwise leached out into the culture medium. One foam slab (55 × 20 × 8 mm) was placed in each flask. 100 ml of the cultivation medium was added into the preculture with the germinated spores. Sterilized $CaCO_3$ (1%w/v) was added initially to avoid pH decrease due to the lactic acid production. Each flask was placed in the incubator shaker after sterilization. The fermentation was carried out under the same conditions: 30°C and 160 rpm for 3 days. LA fermentation by free and PU immobilized *R.oryzae* MTCC 8784 was performed and the kinetic and metabolic parameters of fungal growth and LA production were investigated using direct infusion of 100 g/L starch and agrowastes, ,respectively (Tanyildizi *et al.*, 2012).

Repeated Batch fermentation with immobilized R. oryzae *MTCC 8784 in polyurethane foam*

Four batch cycles of cultivation of the immobilized cells were carried out on a shaker under aerobic conditions (160 rpm, 30°C, 3 days) in 250-ml Erlenmeyer flasks with 100 ml of the starch containing medium. The duration of the first cycle was 48 h, and the next three cycles lasted each for 24 h. The immobilized fungus cells were washed with sterile distilled water between subsequent batch cycles and transferred to fresh medium containing starch (100g/L). To maintain pH at the optimal level, $CaCO_3$ (10 g l^{-1}), preliminarily sterilized in a dry form, was added to the nutrient medium during every batch. A total of 4 batch cylces of fermentation was performed.

DNS method for reducing sugar estimation

Total reducing sugars in the fermentation broth was determined by Miller method using dinitrosalicylic acid reagent (Miller 1959).

Titratable acidity

Every third day, unless otherwise stated, the flasks were removed and the fermentation product was centrifuged at 8000 x g for 10 min. The acidity as total acidity in the fermentation broth was determined by titrating the samples with 0.1N NaOH according to the method given by AOAC (2000). Every 1ml of 0.1N NaOH is equal to 0.009 g of Lactic acid.

Colorimetric estimation of Lactic acid

The concentration of lactic acid was measured based on colorimetric determination (Kimberly and Taylor, 1996). In this method, known amounts of production medium were taken during fermentation and centrifuged at 8000 x g for 10 min. The supernatant was used directly for determination of lactic acid concentration. Assay mixture containing 0.5 ml of supernatant (10-50 mg/l lactic acid) were well mixed with 3 ml of sulfuric acid and heated in boiling water bath. In this method, acetaldehyde is released from lactic acid by hot sulfuric acid effects. After 10 min, the mixture was cooled at room temperature and mixed with 50 µl of $CuSO_4$ (4% in distilled water) and 100 µl of ρ-Phenyl phenol reagent (1.5% in ethanol 95%). The acetaldehyde reacts with copper and ρ-phenyl phenol and produces a chromogenic complex. After 20 min, the absorbance was read against blank at 570 nm colorimetrically.

High pressure liquid chromatography analysis

The qualitative analysis of lactic acid was analyzed using reverse phase high pressure liquid chromatography (HPLC) (Domínguez and Vázquez, 1999). HPLC analysis for lactic acid excreted from the metabolic activities of *R. oryzae* MTCC 8784 were performed on a Waters 518 model series comprised of a quaternary pump with auto-sampler injector, micro-degassers, column compartment equipped with thermostat and a diode array detector. The column used was a C18 (Waters 518) end capped 5 µm, 4.8x250 mm reverse phase column. The mobile phase used was acetonitrile: water (7:3 v/v). Flow rate was adjusted to 1.0 mL/min for 15 min. The temperature of the column was maintained at 25°C. 1 ml of fermentation sample was taken after 72 h of fermentation, centrifuged at 14,000 rpm for 10 min in Remi centrifuge in order to separate the cell mass and other insoluble materials. Supernatants were diluted 10 times to get more precise results from HPLC. Samples and standard (Sigma, USA) (10 µl) were injected using an autoinjector. Lactic acid was detected at 210 nm by the 410 Water UV detector.

Lactic acid yield

Lactic acid yield in the fermentation broth using starch was determined by the following formula

$$\text{Lactic acid yield} = \frac{\text{Lactic acid concentration (g/L)} \times 100}{\text{Initial starch concentration (g/L)}}$$

Theoretically, one gram of starch can be converted to 1.11 g lactic acid (Huang *et al.*, 2005).

Statistical analysis

The optimization experiments were carried out in triplicate. The data was analyzed using MS Excel and SPSS-ANOVA. All data sets were expressed along with their mean standard deviation.

Results and Discussion

Screening of different raw materials for reducing sugar content

The main goal of any pre-treatment, is to alter or remove structural and compositional impediments to hydrolysis of lignocellulosic biomass and subsequent degradation processes in order to enhance digestibility, improve the rate of enzyme hydrolysis and increase yields of intended products (Hendriks and Zeeman, 2009). These methods cause mechanical, physical chemical or biological changes in the plant biomass in order to achieve the desired products. *R oryzae* is also known to produce fumaric acid from agrowaste substrate. Presence of lactic acid was confirmed by estimation using p-hydroxy diphenyl and HPLC methods (Domínguez and Vázquez, 1999). However

Table 1. Biomass of *R. oryzae* MTCC 8784 from pretreated agrowastes (100 g/L)

S.No.	Agrowastes (100g/L)	Steam explosion (g)	Acid hydrolysis (g)	Direct hydrolysis (g)
1.	Sugarcane bagasse (SB)	1.3^{a}	1.2^{ab}	1.18^{a}
2.	Rice bran (RB)	1.19^{b}	1.26^{a}	1.15^{b}
3.	Ground nut oil cake (GOC)	1.19^{b}	1.2^{ab}	1.13^{c}
4.	Wheat Bran (WB)	1.17^{b}	1.21^{ab}	1.13^{c}
5.	Ragi bran (RaB)	1.14^{b}	1.15^{b}	1.09^{e}
6.	Coconut Oil Cake (COC)	1.19^{b}	1.14^{b}	1.09^{e}
7.	Tea waste (TW)	1.12^{c}	1.16^{b}	1.11^{d}
8.	Starch (Control)	1.06^{c}	1.12^{b}	1.05^{f}

Values are the means of triplicate ±SD ($p \leq 0.05$).
Means followed by same letter in each column do not differ significantly by DMRT ($p<0.05$)

Table 2. Lactic acid yield of *R. oryzae* MTCC 8784 from pretreated agrowastes (100 g/L)

S.No.	Agrowastes (100g/L)	Steam explosion (g/L)	Acid hydrolysis (g/L)	Direct hydrolysis (g/L)
1.	Sugarcane bagasse (SB)	76.5^{a}	79.4^{a}	72^{a}
2.	Rice bran (RB)	74.2^{b}	78.27^{ab}	71.43^{a}
3.	Ground nut oil cake (GOC)	74.87^{ab}	77.133^{bc}	70.4^{b}
4.	Wheat Bran (WB)	73.9^{b}	78.133^{ab}	68.63^{c}
5.	Ragi bran (RaB)	74.27^{b}	72^{d}	68.4^{c}
6.	Coconut Oil Cake (COC)	72^{c}	76.5^{c}	69.56^{b}
7.	Tea waste (TW)	68.97^{d}	69.67^{e}	68.66^{c}
8.	Starch (Control)	69.133^{d}	71^{d}	66.6^{d}

Values are the means of triplicate ±SD ($p \leq 0.05$).
Means followed by same letter in each column do not differ significantly by DMRT ($p<0.05$)

for routine estimation, lactic acid was estimated in terms of titratable acidity.

Lactic acid production from different agrowastes

The production of lactic acid was primarily detected by estimating the titrable acidity of the fermentation medium on daily basis, by titrating the fermentation medium against 1N NaOH. All the 3 treatments supported both fungal biomass and lactic acid production (Table 1 and 2). Of the three pretreatments, acid hydrolysis supported higher lactic acid production from all agrowastes. Among all the agrowastes tested by all three pre-treamtents, sugar bagasse supported significantly higher ($P \leq 0.05$) lactic acid yield on all three pre-treatments with acid hydrolysis of bagasse supporting significantly highest ($P \leq 0.05$) lactic acid yield of 79.2 g/L.

Low cost raw materials, such as starchy and cellulosic materials, whey, and molasses, have been used for lactic acid production (Hofvendahl and Hahn-Hagerdal, 2000). Among these, starchy and cellulosic materials are currently receiving a great deal of attention, because they are economical, abundant, and renewable (Oh *et al*., 2005). The starchy materials used for lactic acid production include sweet sorghum, wheat, corn, cassava, potato, rice, rye, and barley (Yun *et al*., 2004). Cellulosic materials have been used for lactic acid production in similar ways as starchy materials. These materials consist mainly of β (1,4)-glucan, and often contain xylan, arabinan, galactan, and lignin. Previous reports indicate production of lactic acid from pure cellulose through simultaneous saccharification and fermentation (SSF) (Yanez *et al*., 2003). The utilization of corncob, waste paper and wood, has been reported as well (Miura *et al*., 2004). Sreenath *et al.* (2001) investigated the production of lactic acid from agricultural residues such as alfalfa fiber, wheat bran, corn stover, and wheat straw. Garde *et al.* (2002) used hemicellulose hydrolyzate from wheat straw for lactic acid production by co-culture of *L. brevis* and *L. pentosus*.

Sugarcane bagasse, a byproduct of the sugar industry, is an abundant source of lignocellulose. Although it is used as fuel for boilers, a large quantity of this material is accumulated in sugar processing

Table 3. LA production immobilized *R. oryzae* MTCC 8784 starch and agrowastes (100 g/L)

Agrowastes (100g/L)	Lactic acid yield (g/L)	
	Free cells	PU immobilized
Sugarcane bagasse (SB)	72[a]	88.4[a]
Rice bran (RB)	71.43[a]	87.13[b]
Groundnut oil cake (GOC)	70.4[b]	84.23[d]
Wheat bran (WB)	68.63[c]	85.2[c]
Ragi bran (raB)	68.4[c]	81[e]
Coconut oil cake (COC)	69.56[b]	77.47[g]
Tea waste (TW)	68.66[c]	79[f]
Starch (Control)	66.6[d]	77[g]

Values are the means of triplicate ±SD ($p \leq 0.05$). Means followed by same letter in each column do not differ significantly by DMRT ($p<0.05$)

plants, which leads to environmental problems (Sasaki *et al.*, 2005). Advanced utilization of this material as a carbon resource holds promise for the bioproduction of useful chemicals like lactic acid.

Immobilization studies

Cultivation of polyurethane sponge immobilized *R.oryzae* MTCC 8784 on starch at 30°C, 160 rpm of agitation rate and pH of 6.0 for 72 h, showed 1.15 fold increase in LA concentration with 1.14 fold increase in volumetric productivity as compared to free cells (Table 3). Table 3 also summarizes lactic acid production by free and immobilized *R.oryzae* MTCC 8784 using starch (100g/L) and direct infusion of agrowastes (100g/L) as substrate. All the agrowaste substrates equally supported lactic acid yield significantly higher ($P \leq 0.05$) than with free cells from the same substrates using PU immobilized *R.oryzae* MTCC 8784 at 30°C, 160 rpm of agitation rate and pH of 6.0 for 72 h with direct hydrolysis of sugar bagasse showing a significantly high ($P \leq 0.05$) yield of 88.2g/L (1.22 fold higher) compared with fermentation using free cells of *R.oryzae* MTCC 8784 on bagasse.

In a similar fashion, maximum lactic acid production of 93.2 g/L was obtained using a glucose concentration of 150 g/l, pH 6.39 and agitation rate 147 rpm, using PU immobilized *R.oryzae*, about 55% higher than production of lactic acid from suspension culture systems (Tanilyadzi *et al.*, 2012). A maximal process productivity of 1.69 g/L, maximal lactic acid concentration of 42.19 g/L and average yield coefficient of 0.96 g/g were achieved in repeated batch fermentation on the liquid stillage without mineral or nitrogen supplementation using *L. rhamnosus* immobilized on zeolite (Djukic´-Vukovic *et al.*, 2013). Several researchers have attempted to use immobilization techniques for L(+)-lactic acid production with *R. oryzae*. The entrapment methods using soft gels such as Ca-alginate have mostly been employed in these studies (Hang *et al.*, 1989). In gel-entrapping methods, the limitation of oxygen supply because of diffusional resistance might decrease the fermentation rate and/or L(+)-lactic acid transformation efficiency (Dong *et al.*, 1996). The problems, associated with filamentous fungal fermentations can be overcome with cell immobilization on support polymer matrix. In this work, the cells were immobilized by physical entrapment in the open pore network of reticulated polyurethane foam which provides less diffusional resistance to substrate transfers. Spores would enter the loose matrices and grow inside the cubes. Then the mycelia will be embraced by the matrices after growing up. Immobilization of cells has been one of the means for high cell retention in the bioreactor. Several materials, such as Ca-alginate gels, poly (ethyleneimine), and plastic composite support, have been used for immobilization of LAB in order to produce lactic acid (Zhang *et al.*, 2007). During the extraction of lactic acid, organic solvents could damage bacterial cells and they have also a negative effect on animal health (Djukic´-Vukovic *et al.*, 2013). For these reasons, immobilization could be a promising strategy for easier separation of the biomass from fermentation media. In the immobilization by adsorption, the biocatalysts are held to the surface of the carriers by physical forces (Van der Waal's forces). The advantages of this method are simplicity, minor influence on conformation of the biocatalyst and there is no need for utilization of chemicals which could cause a damage of bacterial cells, so the catalytic activity could be preserved (Tay and Yang, 2002).

Repeated Batch cycles for lactic acid production using immobilized R.oryzae *MTCC 8784*

Cultivation of polyurethane sponge immobilized *R.oryzae* MTCC 8784 using starch (100 g/L) at 30°C, 160 rpm of agitation rate and pH of 6.0, lactic acid of 54 g/L was obtained after 48 h cultivation. Efficiency on lactic acid production using starch by polyurethane sponge immobilized *R. oryzae* MTCC 8784 showed lactic acid production upto 4 batches tested with 54 g/L of lactic acid in the first batch (48 h), 56.7 g/L by the second batch culture, third batch yielded 59.4 g/L and the fourth batch yielded 61.2 g/L of lactic acid (Figure 2).

Lactic acid detection by HPLC

The retention time for lactic acid was around

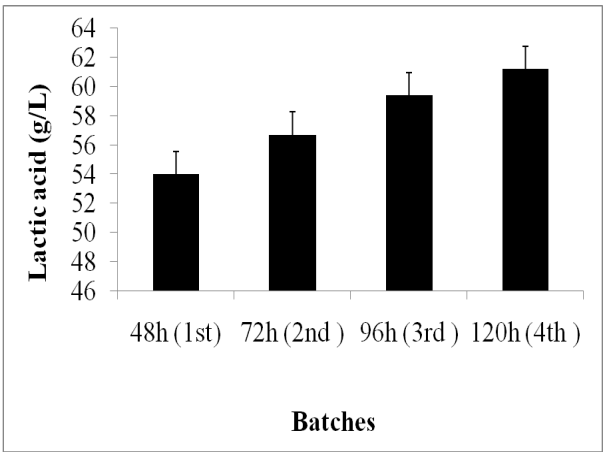

Figure 1. Repeated batch cycles of immobilized *R. oryzae* MTCC 8784 for L-lactic acid production using starch (100 g/L). Values are the means of triplicate ±SD ($p \leq 0.05$)

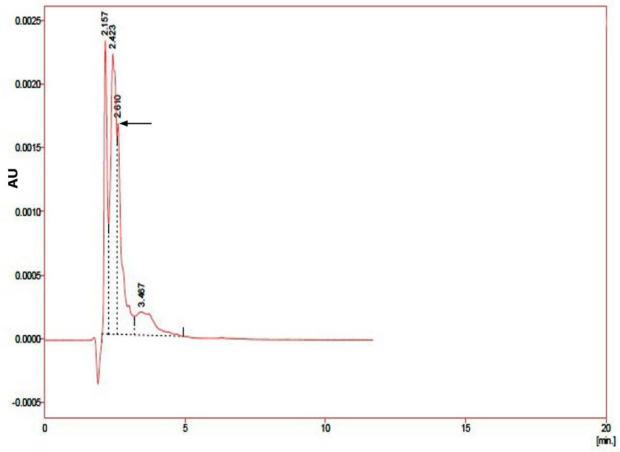

Figure 2. HPLC chromatogram of L-Lactic acid produced by *R. oryzae* MTCC 8784. Arrow indicates the peak of lactic acid supeimposed with standard lactic acid

2.61 minutes which overlapped with the lactic acid standard (2.65 mins) (Fig. 3). Other peaks indicate presence of other organic acids like citric acid and fumaric acid, as *R.oryzae* is a heterofermentative organism. *R. oryzae* produces mainly lactic acid from glucose with yields of 60–80% and also ethanol, carbon dioxide and minor amounts of malic acid, fumaric acid and citric acid (Skory 2004). Product formation depends on cultivation conditions; it has been shown that, under oxygen-limiting conditions, product formation shifts from lactic acid to ethanol (Taherzadeh *et al.*, 2003).

As a non-grain raw material, sugar bagasse does not compete with grain as an agricultural crop, and the use of bagasse has no impact on the food chain for humans. In the present study, we took L-lactic acid as an example for bulk chemical production from sugarcane bagasse, a waste and inexpensive by-product. It may provide an economic L-lactic acid production process with inexpensive and renewable biomass. In this work, the cells were immobilized by physical entrapment in the open pore network of reticulated polyurethane foam which provides less diffusional resistance to substrate transfers. Spores would enter the loose matrices and grow inside the cubes. Then the mycelia were embraced by the matrices after growing up (Dong *et al*., 1996). In a similar work, lactic acid production from *R.oryzae* MTCC 8784 which was immobilized on polyurethane foam matrix with agro-industrial waste like sugar bagasse was found about 1.22 fold higher than lactic acid production obtained in the medium where free cells were used (Bulut *et al.*, 2004).

Conclusion

The study explored the potential of using agrowastes for lactic acid production by *R.oryzae* MTCC 8784 under free and immobilized conditions. The innate enzyme hydrolysate of all the substrates supported lactic acid production comparable to the yield obtained by other pre-treatments. PU immobilized system using starch improved the lactic acid yield and also recycle feasibility upto four cylces. Improved lactic acid yield by the strain was obtained with PU immobilized *R.oryzae* MTCC 8784 with agrowastes as substrates with sugar bagasse supporting the highest yield both under free and immobilized conditions.

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