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Production of L-Lactic acid from starch and food waste by amylolytic *Rhizopus oryzae* MTCC 8784

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Abstract: Lactic acid, commonly used in food, chemical and pharmaceutical industries, has recently received much attention for the production of biodegradable plastics. In this study, *Rhizopus oryzae* MTCC 8784 was used to convert starch into optically pure L-Lactic acid. Effect of time, pH, addition of calcium carbonate and starch concentration were analyzed using shaker flasks in order optimize the L-lactic acid concentration and volumetric productivity. Under optimized conditions [pH6.0, $CaCO_3$ (10g/L), starch (10g/L) and 72 h of cultivation] *R.oryzae* MTCC 8784 gave a lactic acid concentration of 50.48g /L with a volumetric productivity (Q_p) of 0.7 (g/L/h) and lactic acid yield($Y_{P/S)}$ of 4.54 g/g (2 fold increase), 100% starch saccharification, and 1.5 g/L mycelial biomass. To reduce the Lactic acid production costs, inexpensive raw materials such as food waste (fruit and vegetable peel/waste) like sapota, banana, papaya, potato, corn cob and carboxymethyl cellulose were explored. All substrates tested supported growth and lactic acid production. Efficient lactic acid concentration (72g/L) with a biomass of 1.9 g/L; volumetric productivity (Q_p) of 1g/L/h and lactic acid yield($Y_{P/S)}$ of 3.6g/g was obtained with sapota peel fermentation. Lactic acid was detected in the fermentation broth by HPLC.
Key words: *R.oryzae* MTCC 8784, lactic acid, process optimization, starch, vegetable and fruit waste.

Introduction

Lactic acid is a valuable chemical and one of its extensive applications is for polymerization of L-lactic acid to poly (L-lactic acid), which is an attractive polymer because it can be produced from renewable resources and is biodegradable. These properties have strengthened

interest in developing more efficient production processes for optical purity of L-lactic acid[1]. In India, the annual production capacity of Lactic acid is 6000t and an estimated gap of 2300 t in supply by the year 2015 have been predicted, if the present level of production is not increased[2]. Wastes containing starch generated from food processing plants may be regarded as a viable option for meeting this growing demand for lactic acid, if appropriate biotechnological interventions are used and specific sectors amongst the Indian food processing industry are targeted[3]. Rapidly increasing prices of animal feed supplements is one of the challenges

faced by livestock industries at a global scale. Incidentally, solid unwanted agricultural materials resulting from postharvest activities of farmers and food processors are also growing at a faster rate due to improved farming methods and high fruit and vegetable produce[4]. Therefore, fruit and vegetable residues remain source of solid agricultural waste. Fruit residues may cause serious environmental problems, since it accumulates in agro-industrial yards without having any significant and commercial value. Since disposal of these wastes is expensive due to high costs of transportation and a limited availability of landfills they are unscrupulously disposed causing concern as environmental problems. Furthermore, the problem of disposing by-products is further aggravated by legal restrictions. A high level of BOD and COD in fruit wastes add to further difficulties in disposal. However, inspite of their pollution and hazard aspects, in many cases, food processing wastes have a good potential for conversion into useful products of higher value as by-product, or even as raw material for other industries. A large quantity of the waste include peels, seeds and pulps depending on the type of fruits[5]. Banana waste (peel), pineapple waste and papaya waste are examples of agricultural wastes found abundantly in several tropical and sub-tropical areas such as Indian subcontinent and Southeast Asian countries[6].

Unlike the Lactic acid bacteria (LAB), lactic acid producing *Rhizopus* strains generate L-lactic acid as a sole isomer of lactic acid[7-10]. The production of L-lactic acid using a surface culture of *Rhizopus* was reported in 1911[11]. An efficient submerged fermentation using fungal species for the production of L-lactic acid was first reported in 1936[12-13]. However, an increased research interest has been given to lactic acid fermentation by fungal species in recent decades[14].

The present study attempted at identifying the process parameters in terms of time, pH, neutralizing agents ($CaCO_3$), starch concentration for maximizing lactic acid production by *R.oryzae* MTCC 8784 starch by submerged fermentation (SmF). The biotechnological conversion of a few vegetable and fruit wastes to lactic acid by *R.oryzae* MTCC 8784 is also demonstrated.

Experimental

Microbial strain

The pure culture of *Rhizopus oryzae* MTCC8784 (Microbial Type Culture Collection, Chandigarh) was obtained and it was cultured on solid Sabouraud's agar. Mycelia of freshly grown culture was used as inoculum for Lactic acid production.

Media and fermentation conditions

The fermentations at shake flask level (50 mL in a 250 mL Erlenmeyer flask) were carried out using the media as described by Huang et al. (2003)[15], and contained carbohydrates 40g/L, yeast extract 10g/L, peptone 20g/L, K_2HPO_4 0.5g/L, KH_2PO_4 0.5g/L, $MgSO_4.7H_2O$ 0.05g/L, $(NH_4)_2SO_4$ 2g/l, pH 6.0, agitation at 160 rpm, temperature at 30°C. Starch was used as carbohydrate source unless otherwise stated. Peptone, yeast extract, ammonium sulphate were used as nitrogen substrates. Magnesium sulphate ($MgSO_4.7H_2O$), dipotassium phosphate (K_2HPO_4), monopotassium phosphate (KH_2PO_4) were used as inorganic minerals.

Agro-Food Waste Substrates

A total of six different types of agro-food wastes *viz.* Sapota peel, banana peel, papaya peel, potato peel, corn cob powder and carboxymethyl cellulose (CMC) (procured from the local markets of Bangalore), and the material from one single batch was used in all the studies in order to minimize any possible interference due to variation in composition of residues. The samples were dried in an oven at 60°C for two days, grounded and screened to collect the particles of the size between 1.2 and 1.6 mm. Steam explosion treatment was given to the substrates, amount of sugar content was determined by preparing their hydrolysate and used for lactic acid production by *R.oryzae* MTCC 8784.

Steam explosion

The modified method of Pumiput *et al*. (2008) was used for substrate hydrolysate preparation[16]. 40 g of each agro-food waste was steam-exploded in 100 L capacity autoclave at 121°C for 20min. Water was added to the wet pre-treated material to make up the volume of 1 L and boiled at 80°C for 30 min. Later the hydrolysate was recovered by filtration with cheese cloth.

Acid Hydrolysis

Acid post hydrolysis of hemicellulose hydrolysate was carried out to cleave the xylooligosaccharides into monomeric sugars by autoclaving at 121°C with concentrations of HCl varied from 2% v/v for 30 min[16]. Chemical pre-treatment is to remove chemical barriers, so the enzymes can have access to cellulose for microbial destruction. The hydrolysate from acid post hydrolysis was adjusted with CaO to pH 6- 6.8 and the CaSO4 precipitates were removed by filtration with Whatmann filter paper No.1[15].

Direct hydrolysis

Direct infusion was carried out by drying and pulverizing the agro-wastes. The powdered substrates were added as the sole carbon source and the media was supplemented with minimal salts.

Analytical methods

Screening for Lactic acid production

Czapeck dox agar (HIMEDIA, INDIA) plates with bromocresol green (0.05%, w/v) as the indicator or supplemented with 1% $CaCO_3$ were inoculated with a loopful of fungi and incubated for three to five days for the formation of either yellow zone or clearance around the mycelial growth, respectively, indicating the presence of Lactic acid production.

DNS method for reducing sugar estimation

Total reducing sugars in the fermentation broth was determined by Miller method using dinitrosalicylic acid reagent[17].

Starch-Iodine method for residual Starch estimation

The method developed by Tomas and Chamberlain (1980)[18] was used which is based on color development that results from iodine binding to starch polymers. The initial as well as residual starch was estimated by using this method.

Titratable acidity

Every third day, unless otherwise stated, the flasks were removed and the fermentation product was centrifuged at 8000 x g for 10mins, the centrifuged supernatant was heated at 70°C , to this 5% $Ca(OH)_2$ was added and filtered with Whatman filter paper1, the precipitant was collected and dissolved with 0.1N HCL and then titrated with 1M NaOH. The acidity as total titrable acidity was determined titrating the samples with 1N NaOH according to the method given by AOAC (2000)[19]. Every 1ml of of NaOH is equal to 90.08 mg of Lactic acid.

HPLC analysis

The qualitative analysis of lactic acid was analyzed using reverse phase high pressure liquid chromatography (HPLC)[20]. HPLC analysis for lactic acid excreted from the metabolic activities of *Rhizopus* 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 eluant used was acetonitrile : water (7:3 v/v) and the column separation was allowed at a flow rate of 1.0 mL/min for 15 minutes. 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 minutes 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 standards (10µl) were injected using an autoinjector. Lactic acid was detected at 210nm by the 410 Water UV detector.

Results and Discussion

R.oryzae MTCC 8784 produced organic acid as revealed by the yellow coloration of Bromocresol indicator plate (Plate 1 c & d) and precipitation of $CaCO_3$ (Plate 1 a & b). The one factor at a time is the most frequently used operation in optimization process. This technique involves changing one independent variable while keeping the other factors constant. In the present study, we described optimized fermentation medium and conditions [time, pH, $CaCO_3$, starch] to obtain maximum lactic acid production with *Rhizopus oryzae* MTCC 8784.

Plate 1. Plate 1: Screening of *R.oryzae* MTCC 8784 for acid production using $CaCO_3$(1%,w/v) plates a) Control and b) Growth of *R.oryzae* showing precipitation of $CaCO_3$; Bromocresol plates (0.05%,w/v) c) Control plate and d) Growth of *R.oryzae* showing yellow coloration

Time course production of L-Lactic acid by *R.oryzae* MTCC 8784

The biochemical kinetics of simultaneous saccharification and fermentation (SSF) for lactic acid production by fungal species of *Rhizopus oryzae* 8784 was studied with respect to time of incubation.

Rhizopus oryzae MTCC 8784 produced 50.48 g/L of L-lactic acid by 72h from starch (10g/L) (Fig.1). The fungal biomass gradually increased from day one (1.2g/L) to day three (1.6g/L) with concomitant utilization of starch and then remained constant. By day five, starch was completely utilized. The production of L-lactic gradually increased from day one (19.97g/L), peaked by day three (50.48g/L) and the production gradually decreased on day four and five.

Fungal *Rhizopus* species have attracted a great interest, and have been recognized as suitable candidates for lactic acid production. *Rhizopus oryzae* can produce large amounts of L-lactic acid and utilize both various sugars and starch as carbon sources[8-10]. Lactic acid production using *Rhizopus oryzae* seems to be a viable alternative because it can grow on minimal liquid medium and on solid medium[7].

Starch has been considered for use as a raw material for various fermentation because of its abundance and low price. However, when high concentration of starch is used in medium, an increase in viscosity of the medium due to gelatinization by heat will reduce the microbial growth.

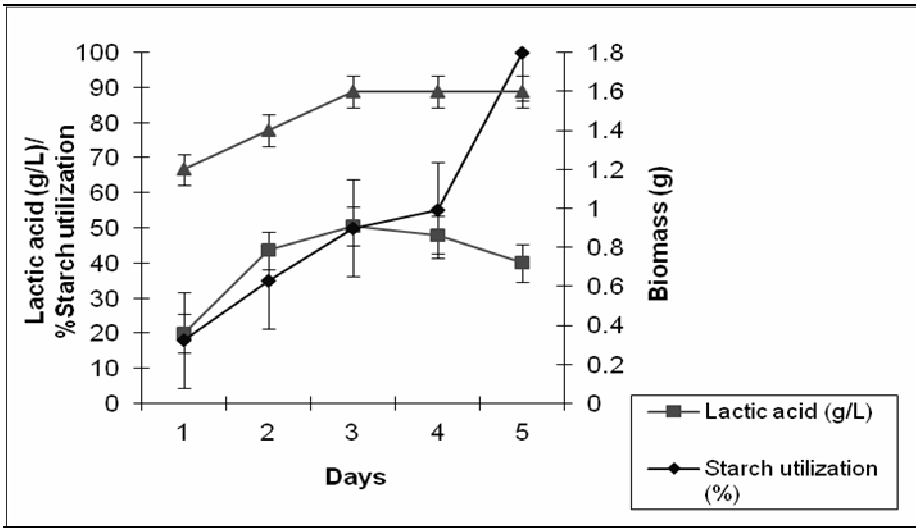

Fig. 1. Time course production of L-Lactic acid by *R.oryzae* MTCC 8784.

Effect of growth pH

In order to determine the impact of growth pH (uncontrolled) on the starch saccharification and fermentation of lactic acid by the *R. oryzae* MTCC8784, in absence of $CaCO_3$, the initial growth pH was set at 4.0, 5.0, 6.0, 7.0 and 8.0 by adding 4N NaOH solution.

The time course study proved that the fungus species demonstrated the highest lactic acid production by 72 h, the variations in starch, reducing sugar concentrations and lactic acid yield in the culture were measured on day 3 (Fig. 2).

The experimental results revealed that *R. oryzae* MTCC 8784 had a metabolic capability to saccharify starch, produce lactic acid and fungal biomass using starch (20g/L) with a pH range between 4.0 and 7.0 with maximum growth, starch utilization and lactic acid yield at pH 6.0 (with the residual pH dropped to pH 4.5), after which all these parameters were affected at pH 7.0 and pH 8.0. Similar observations have been reported by Domínguez and Vázquez (1999)[20] for *R.oryzae* wherein pH 3.5-6.0 supported lactic acid production.

Effect of supplementation of $CaCO_3$

Lactic acid is known to be a strong inhibitor for both cell growth and lactic acid production [21].Calcium carbonate is a commonly used reagent to neutralize lactic acid during fermentation. Its low solubility in water makes it possible to neutralize lactic acid and maintain the pH at certain level automatically[22].

Addition of $CaCO_3$ in the concentration of 0.5, 1.0, 1.5 and 2.0% (w/v) did not have any profound effect on biomass, starch utilization and lactic acid production as compared to the control set at an initial pH 6.0 without any $CaCO_3$ addition in the fermentation broth (Fig.3). This indicates that *R oryzae* MTCC8784, addition of $CaCO_3$ 1% (w/v) was found to be sufficient for maintaining a growth pH for one batch, to achieve an optimum fungal cell growth and lactic acid production.

Soccol et al., (1994)[7] demonstrated that the addition of $CaCO_3$ increases the lactic acid production from concentrations lower than 3g $litre^{-1}$ to 65g $litre^{-1}$. Other chemicals have also been recommended. Friedman & Gaden, (1970)[23] proposed the use of sodium hydroxide meanwhile Stieber & Gerhardt (1995)[24] worked with ammonium hydroxide. In a study by Huang et al., (2005)[21] on Simultaneous saccharification and fermentation of potato starch wastewater to lactic acid by *Rhizopus oryzae* and *Rhizopus arrhizusi*, $CaCO_3$ addition of 1% (w/v) was found to be sufficient for maintaining a growth pH to achieve an optimum fungal cell growth and lactic acid production using potato starch in both *Rhizopus* cultures. This is in contrast to our results.

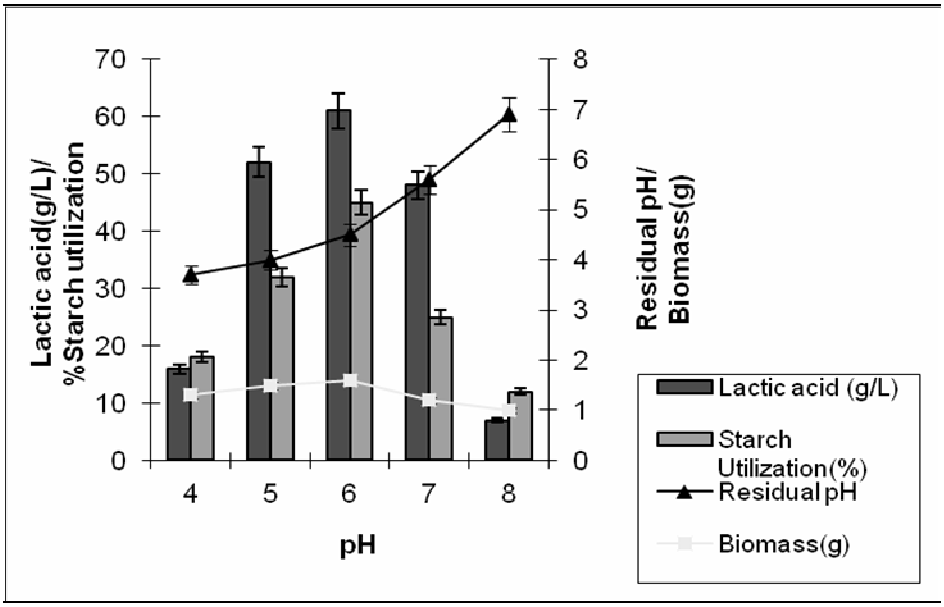

Fig.2. Effect of initial media pH on LA production by *R.oryzae* MTCC 8784.

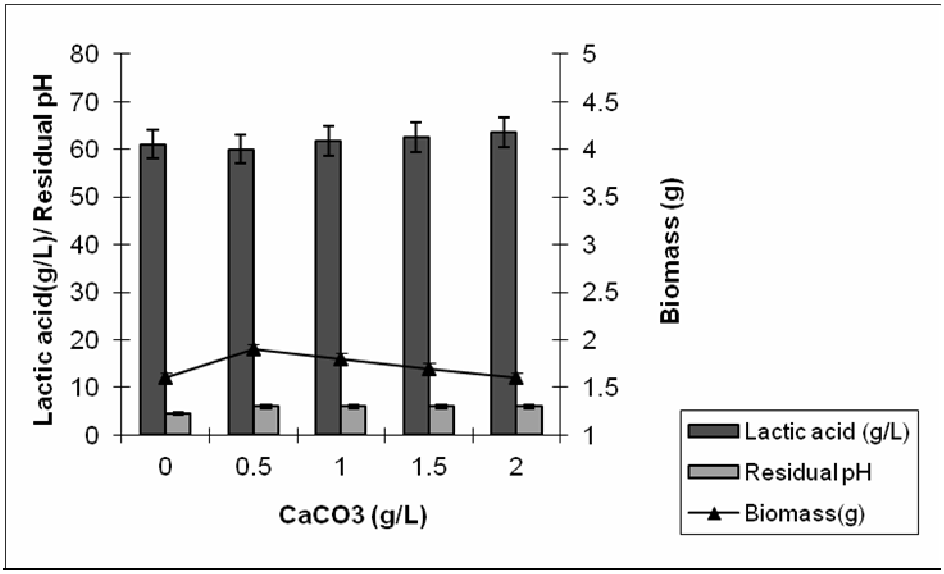

Fig.3. Effect of $CaCO_3$ supplementation on LA production by *R.oryzae* MTCC 8784.

Effect of starch concentration

The effect of starch concentration varying from 10 to 50 g/l in submerged fermentation was investigated, and the results are shown in Fig.4. The concentration of lactic acid produced by *R. oryzae* MTCC 8784 increased from 50 to 66.6 g/l with increasing initial starch concentration from 10 to 40 g/l with a concomitant increase in biomass from 1.3g/L to 1.6g/L (Fig.4). A further increase in initial starch concentration (50g/L) resulted in a gradual decrease in lactic acid production (64.8g/L) and biomass (1.5g/L). Starch was completely utilized in all the concentrations indicating good amylolytic potential of the strain.

Decrease in yield with further increase in starch concentration beyond 40g/L may be due to substrate inhibition during later stages of fermentation at high concentration of substrates.

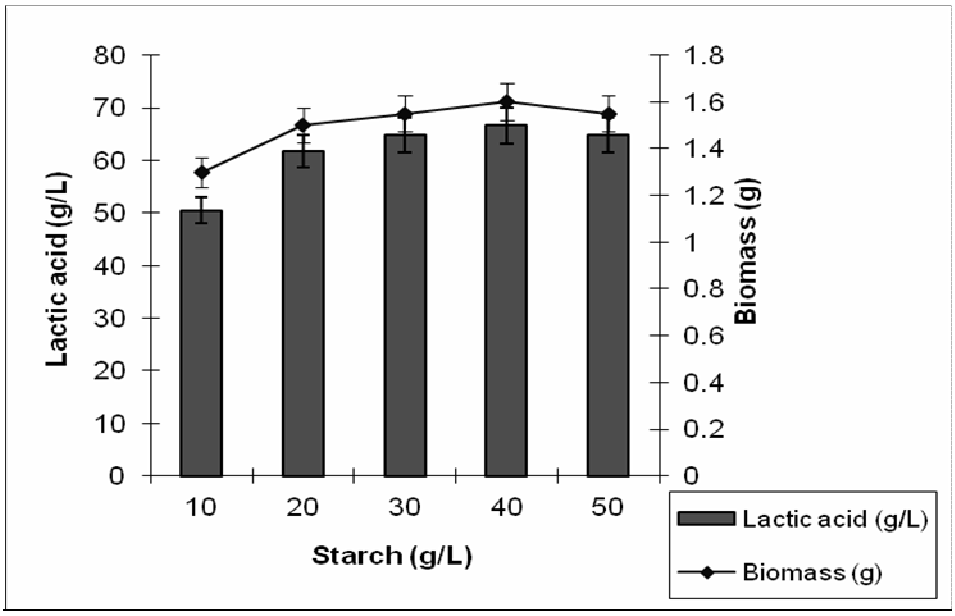

Fig.4. Effect of starch concentration on LA production by *R.oryzae* MTCC 8784.

Kinetic characteristics of lactic acid production

The investigation of kinetic characteristics in the SmF process was conducted in shake flask fermentation using starch (20 g/l). Cultivation conditions were set up at an initial pH of 6.0 with supplementation of 1% (w/v) $CaCO_3$ at 30°C. Samples were taken at the end of 72 h fermentation. Table 1 summarizes the kinetics of LA production by *R.oryzae* MTCC 8784 under un-optimized and optimized conditions.

Under optimized conditions [pH6.0, $CaCO_3$ (10g/L), starch (10g/L) and 72 h of cultivation] *R.oryzae* MTCC 8784 gave a lactic acid concentration of 50.48g /L with a volumetric productivity (Q_p) of 0.7 (g/L/h) and lactic acid yield ($Y_{P/s)}$ of 4.54 g/g (2 fold increase), 100% starch saccharification and 1.5 g/L mycelial biomass.

Lactic acid detection by HPLC

The retention time for lactic acid was around 2.61 minutes which overlapped with the lactic acid standard (2.65 mins) (**Fig.5).** 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[25,26]. Product formation depends on cultivation conditions; it has been shown that, under oxygen-limiting conditions, product formation shifts from lactic acid to ethanol[26,27].

Table 1. Comparison of fermentation kinetics of LA production by *R.oryzae* MTCC 8784 under un-optimized and optimized conditions of cultivation by SmF.

Condition	Starch (g/L)	Lactic acid concentration (g/L)	Volumetric productivity of Lactic acid (g/L/h)	Lactic acid yield ($Y_{P/S}$) (g/g)
Unoptimized	20(~22.2g glucose)	50.48	0.7 (72h)	2.27
Optimized	10(~11.1g glucose)	50.48	0.7(72h)	4.54

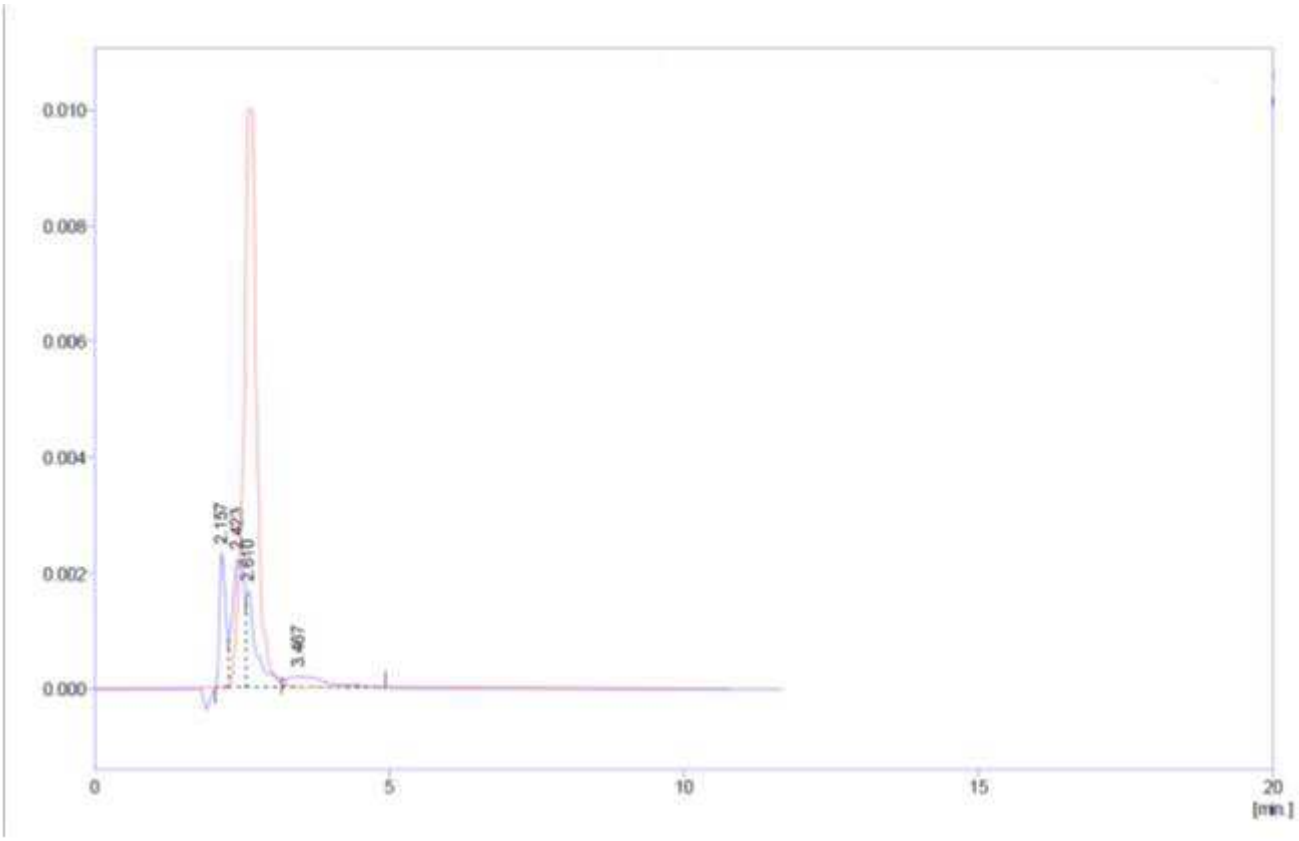

Fig.5. HPLC chromatogram of L-Lactic acid produced by *R.oryzae* MTCC 8784.

Lactic acid production from different agro-food wastes

All complex substrates tested supported appreciable growth and lactic acid production by *R.oryzae* MTCC 8784 (Fig.6). Highest lactic acid concentration (72g/L) with a biomass of 1.9 g/L; volumetric productivity (Q_p) of 1g/L/h and lactic acid yield ($Y_{P/S}$) of 3.6g/g was obtained with sapota peel fermentation followed by corn cob powder [70.2g/L; Q_P (0.98g/L/h); $Y_{P/S}$ (3.51g/g) and biomass (1.7g)].

Cheap raw materials, such as starchy and cellulosic materials, whey, and molasses, have been used for lactic acid production[28]. Among these, starchy and cellulosic materials are currently receiving a great deal of attention, because they are cheap, abundant, and renewable[29]. The starchy materials used for lactic acid production include sweet sorghum, wheat, corn, cassava, potato, rice, rye, and barley[30]. These materials have to be hydrolyzed into fermentable sugars before fermentation, because they consist mainly of α (1,4)- and α (1,6)-linked glucose. This hydrolysis can be carried out simultaneously during fermentation with amylase-producing *L. amylophilus* and *L. amylovorus* which are often used for the direct fermentation of starchy materials into lactic acid[31]. 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. Venkatesh (1997) and Yáñez et al. (2005) have previously attempted to produce lactic acid from pure cellulose through simultaneous saccharification and fermentation (SSF)[32,33]. The utilization of corncob, waste paper and wood, has been reported as well[34]. Sreenath *et al.* (2001) investigated the production of lactic acid from agricultural residues such as alfalfa fiber, wheat bran, corn stover, and wheat straw[35]. They suggested that, during SSF of alfalfa fiber, lactic acid production was enhanced by adding pectinase and cellulase together. Garde *et al.* (2002) used hemicellulose hydrolyzate from wheat straw for lactic acid production by co-culture of *L. brevis* and *L. Pentosus*[36].

Lactic acid has also been produced from waste sisal stems[37], sugarcane bagasse[38] and kitchen waste[39] by using *Lactobacillus* isolates. Fungal production of lactic acid from pineapple waste resulted in 19.3 and 14.7g/L lactic acid with *Rhizopus arrhizus* and *R. oryzae*[40].

Unlike most bacteria, lactic acid-producing fungi, for example, the genus *Rhizopus*, are capable of efficiently secreting lactic acid[41] and some *Rhizopus* strains may secrete L-lactic acid as the only product[42]. In addition, fungal cultures are tolerant of low pH environments. Consequently, pH maintenance during fungal fermentation is not as stringent as with bacterial cultures[21]. Furthermore, fungal cultures, such as *Rhizopus* spp, are amylolytic and can produce lactic acid from starchy substrates such as potato starch without prior saccharification[43]. Fungi also have advantages such as low nucleic acid contents and high levels of protein. It has been demonstrated that the cost of separating biomass from the spent cultivated broth may be a significant fraction of the total capital and operating costs. In the case of fungal cultivation, the mycelial and pellet forms are easy and inexpensive to harvest. Within the *Rhizopus* genus, *R oryzae* has received the greatest interest, and strain *R oryzae* NRRL 395 has been recognized as one of the most suitable lactic acid producers[30]. *R oryzae* NRRL 395 and *R oryzae* IFO 4707 strains were shown to convert ground corn and potato pulp, respectively, directly to lactic acid. *R oryzae* ATCC 52311 could achieve a lactic acid concentration of 83 g dm−3 with a yield of 0.88 g lactic acid g^{-1} glucose consumed.

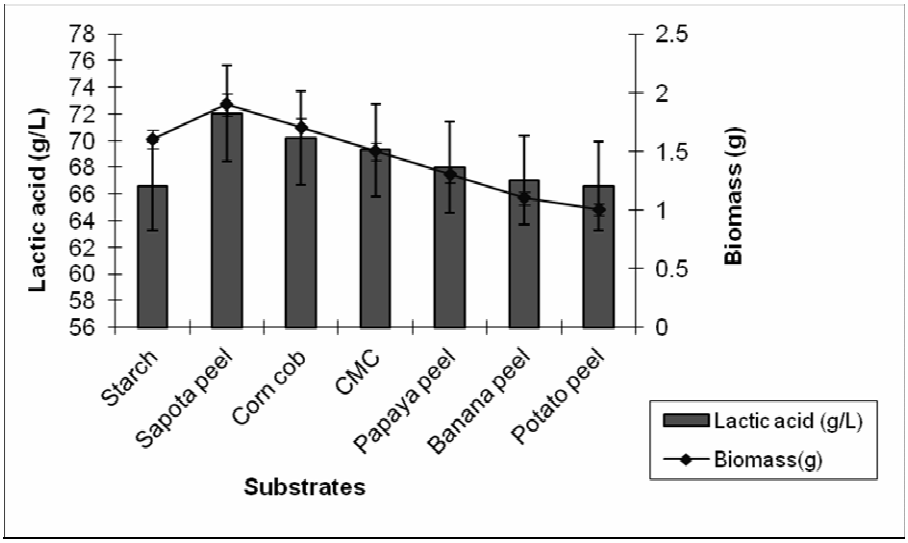

Fig.6. Lactic acid production from agro-food wastes by *R.oryzae* MTCC 8784.

Conclusions

Optimization of process parameters, such as time of incubation, pH, starch concentration and $CaCO_3$ addition resulted in improving performance of *Rhizopus* species in lactic acid production. Lactic acid is known to be a strong inhibitor of cell growth, enzymatic hydrolysis and microbial activity in lactic acid fermentation. To prevent this self-inhibition, the addition of a neutralizing agent is, therefore, necessary. In the present work a significant yield in terms of lactic acid and biomass production was obtained without the addition of 10 g dm^{-3} $CaCO_3$ to the fermentation media for one batch. Hence, this process may alleviate the extra costs for lactic acid purification and biomass recovery.

This study records highest production of lactic acid from sapota peel (72g/L) and corn cob (70g/L) substrates as compared to previous studies which reports around 10.1g/L for corn and 4.2g/L for potato[44]. Also the study shows novel use of food wastes like vegetable and fruit peels as use for substrate in lactic acid production. The inference drawn from this study aims for the use of food wastes for commercial and economic production of application based products by fermentative bioconversions.

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