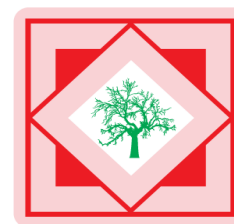




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Simultaneous production of lactic acid and chitin by fungal fermentation

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ABSTRACT

Lactic acid is a colourless liquid which is important both in national and international markets for several industries. It is produced chemically from petroleum feed stocks and biologically from bacterial and fungal fermentation. Chitin is a second abundant biopolymer having wide use in agriculture, textile and biomedical industries and traditionally obtained from marine invertebrates which has some demerits like harsh chemical treatment and protein allergy. In order to alleviate the above problems alternative source fungi was chosen, for its production as chitin is one of the cell wall components of fungi. The present work was designed to produce the above two important products economically by a single fermentation process using fungus *Rhizopus oryzae* NCIM 1009

Keywords: Biomass, Chitin, Fermentation, Lactic acid, *Rhizopus oryzae*

INTRODUCTION

Lactic acid is the most widely utilized organic acid in the food, pharmaceutical, cosmetics and chemical industries. Its production is currently a great deal of research and development [1]. Lactic acid naturally exists in two optical isomers D(-)- Lactic acid and L(+)- Lactic acid. Since elevated levels of D(-)-isomer are harmful to humans L (+)-lactic acid is the preferred isomer in food and pharmaceutical industries. Lactic acid can be produced commercially either by chemical synthesis or fermentation. Chemical synthesis results in racemate mixture of two isomers while the fermentation process can yield an optically pure form of lactic acid or racemate depending upon the microorganisms, substrates and fermentation conditions employed in the production process[2, 3,4].Therefore chemical synthesis may be limited due to a shortage of naturally available raw materials in the future. Lactic acid from biological sources can be produced by both bacteria and fungi fermentation. Generally bacterial fermentation has higher yield[5]. However fungi, *Rhizopus oryzae* have proven to be a good lactic acid producer and has several advantages over bacterial fermentation such as more tolerant to a low pH environment, easy separation of fungal biomass from broth and low nutrition requirements which reduced the fermentation cost and simplifies downstreaming process[6,7].

When lactic acid is produced using *Rhizopus oryzae*, a considerable amount of fungal biomass, whose cell wall is composed of chitin, the second most abundant biopolymer having its wide use in agriculture, textile and biomedical industries, was concurrently produced. Traditionally chitin is produced from marine invertebrates which has some drawbacks such as harsh chemical treatment and causing protein allergy. In the present research work fungal biomass obtained during the production of lactic acid was utilized for chitin production. Thus an economical process of lactic acid and chitin co-production was designed and studied [8,9].

MATERIALS AND METHODS

Culture and Maintenance media

The microorganism used in this study was *Rhizopus oryzae* 1009 and obtained from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune and grown on potato dextrose agar slants at 30°C for seven days. This culture was transferred once a month to a fresh slant. All the cultures were stored at -2°C to -6°C and purity was tested before using.

Cultivation conditions

For fungal cultivation, 1ml spore suspension (10^7 spores ml⁻¹), prepared from a 7-day old slant, were inoculated into 250 ml Erlenmeyer flasks containing 100ml of the fermentation medium consisting of 20 g/L of glucose, 10 g/L of peptone, 1 g/L of yeast extract, 5 g/L of ammonium sulphate, 1 g/L of potassium hydrogen phosphate dibasic, 1 g/L of magnesium sulphate, 0.1 g/L of calcium chloride and 1 g/L of sodium chloride. After inoculation, the fungi was grown in the fermentation broth for an additional two days in a shaking incubator set at 28°C with agitation of 200 rpm, the pH of the medium was maintained between 3-5 throughout the fermentation and the mycelia was harvested by filtration.

Strain Used	Carbon source		Nitrogen source	Mineral salts					Yield(g/L) at suitable harvesting time	
	Dextrose	Maltose	Peptone	K ₂ HPO ₄	MgSO ₄	(NH ₄) ₂ SO ₄	NaCl	CaCl ₂	Chitin at 96 h	Lactic acid at 168 h
<i>Rhizopus oryzae</i> NCIM1009	X		X	X	X	X	X	X	0.78	20
		X	X	X	X	X	X	X	0.66	18

Simultaneous extraction of chitin and lactic acid

a. Chitin isolation

The biomass was recovered from the fermentation medium by filtration (No.1 Whatman) and washed with distilled water until clear filtrate was obtained. The mycelium was then treated with 1M sodium hydroxide (1:30g/ml) and the mixture was autoclaved at 121°C for 15 min. The mixture was subsequently filtered (No.1 Whatman) to sediment the alkali insoluble materials (AIM) and washed with distilled water and ethanol. The washed material was further extracted with 10% acetic acid solution (1:40g/ml) and refluxed at 65°C for 6 h. The resulting slurry was isolated by filtration (No.1 Whatman) yielding an acid insoluble precipitate (containing chitin) and acid soluble supernatant. The chitin was finally washed with distilled water, 95% ethanol and acetone subsequently. It was then air dried. All the experiments were performed in triplicate.

b. Lactic acid extraction

The filtrate of fermentation broth was utilized for lactic acid extraction. In the filtrate, lactic acid was present either in the form of salts or esters which was isolated and separated. The filtrate was acidified with 15 drops of

concentrated sulphuric acid and boiled for 1 h and filtered. To the filtrate 20 ml of diethyl ether was added and shaken for 15 min in a separating funnel. Then the ether layer containing lactic acid was separated and ether was allowed to be evaporated. The obtained lactic acid was then purified using charcoal. All the experiments were performed in triplicate [10].

Determination of growth curve of chitin and Lactic acid

The growth curve of isolated chitin and lactic acid of *Rhizopus oryzae* NCIM 1009 was determined by culturing fungus in the fermentation medium by inoculating 30 ml of spore inoculum in 300 ml of fermentation medium taken in seven 500 ml Erlenmeyer flasks. Each incubation flask was incubated at different periods of time 48, 72, 96, 120, 144 and 168 hours in a rotary shaker. At the end of each incubation period mycelia were harvested from each of the seven flasks and dried. At the same time lactic acid present in the fermentation broth was extracted and purified. The growth curve of isolated chitin and lactic acid after 48, 72, 96, 120, 144 and 168 hours were determined. Three replicate cultures were prepared for each incubation period in a rotary shaker [11].

Optimization Parameters

Lactic acid and chitin production were optimized by varying one factor while keeping all the other factors as constant. The parameters such as carbon source, nitrogen source, pH, speed of agitation were optimized in this study.

a. Carbon source optimization

The carbon source such as dextrose and maltose were used instead of glucose. The nitrogen source and mineral sources were kept as constant during fermentation.

b. Nitrogen source optimization

Nitrogen sources like corn steep liquor and urea were used and keeping carbon and mineral sources as constant in the medium.

c. Optimization of speed of agitation

Agitation speed was optimized by varying shaking speed in a rotary shaker. The shaking speed such as 200 and 250 rpm were utilized and keeping all other factors constant.

d. pH optimization

The optimum pH for the production of lactic acid and chitin were determined by varying the pH ranges from 3-5. The fermentation medium was prepared and adjusted to a final pH such as 3 and 5.

Characterization of Chitin and Lactic acid

Characterization of chitin by IR spectroscopy

Two milligrams of fungal chitin was dried overnight at 60°C and thoroughly mixed with 100 mg of KBr to produce 0.5 mm thick discs. Spectrum was recorded using JASCO FTIR 410

Characterization of lactic acid by IR spectroscopy and Polarimetry

a. Infrared spectroscopy

1ml of lactic acid was taken in a liquid sample cell and the spectrum was recorded using JASCO FTIR 410.

b. Polarimetry

The optical rotation of lactic acid was found by using polarimetry. 25 ml of lactic acid was placed in the sample cell and the polarized light was allowed to pass through it and the plane of polarisation of polarised light was measured.

RESULTS AND DISCUSSION

The amount of dried biomass and lactic acid of *Rhizopus oryzae* NCIM1009 increased with time. The fungal biomass increased rapidly during first 96 h of incubation. Lactic acid content increases until the sugar concentration in the medium depletes. At 168 h of incubation, lactic acid amount was maximum. The decline of the isolated chitin seen in the growth curve might be due to physiological changes in the fungal cell wall. During the exponential phase, the yield of isolated chitin is relatively high due to active growth. With the accumulation of lactic acid in fermentation process, pH value in the medium decreases sharply.

The result in Table1 represents that the exponential phase of the fungus would give the best yield for chitin and the late exponential growth phase of fungus would give the best yield for lactic acid.

The fungus was harvested after particular incubation period and the yield of lactic acid and chitin were determined. Under this study, the harvesting phase was determined for lactic acid and was found to be 96 h for chitin and 168 h for lactic acid respectively. The maximal isolated chitin and lactic acid yield were determined for different carbon and nitrogen sources by harvesting at the end of incubation period and this depends on the fungal strains, mycelia

age and composition. The results were presented in table 2 and 3. Inclusion of maltose as a carbon source and peptone as a nitrogen source produced high yield of chitin (0.66g/l). Inclusion of glucose as a carbon source and corn steep liquor as a nitrogen source produced high yield of lactic acid (26ml/l). The present study indicated that 200 rpm and pH 3 was optimum for the production of lactic acid and chitin (Figure1-4). Profile of both isolated and commercial lactic acid and chitin showed similar IR spectra (Figure.5-8). The optical rotation of isolated lactic acid was determined by polarimetry and was found to be +95.4 and L (+) isomer.

Table1. Yield of isolated chitin and lactic acid from fungus *Rhizopus oryzae* NCIM1009

S.No	Harvesting Period (h)	Product1 (Chitin)g/l	Product2 (Lactic acid)ml/l
1	48	0.21±0.04	3.5±0.8
2	72	0.18±0.03	8.4±0.05
3	96	0.32±0.1	15.8±0.07
4	120	0.23±0.07	10.4±0.1
5	144	0.27±0.1	26.2±0.3
6	168	0.25±0.08	21.3±0.04

Values are mean ±S.E.M (n=3)

Table 2. Comparison of yield of chitin and lactic acid using different carbon sources from *Rhizopus oryzae* NCIM 1009

Strains used	Carbon Source		Nitrogen Source	Mineral Salts					Yield	
	Dextrose	Maltose		Peptone	K ₂ HPO ₄	Mg	NH ₄ cl	Nacl	Cacl ₂	Chitin (g/l) At 96h
<i>Rhizopus oryzae</i> NCIM 1009	✓	x	✓	✓	✓	✓	✓	✓	0.78	20
	x	✓	✓	✓	✓	✓	✓	✓	0.66	18

Table 3. Comparison of yield of chitin and lactic acid using different nitrogen sources from *Rhizopus oryzae* NCIM 1009

Strains used	Nitrogen Source		Carbon Source	Mineral Salts					Yield	
	Urea	Corn steep liquor		Glucose	K ₂ HPO ₄	Mg	NH ₄ cl	Nacl	Cacl ₂	Chitin (g/l) At 96h
<i>Rhizopus oryzae</i> NCIM 1009	✓	x	✓	✓		✓		✓	0.43	26
	x	✓	✓	✓		✓		✓	0.36	20

Figure1 and 2. Comparison of yield of chitin and lactic acid at different speed of agitation from *Rhizopus oryzae* NCIM 1009

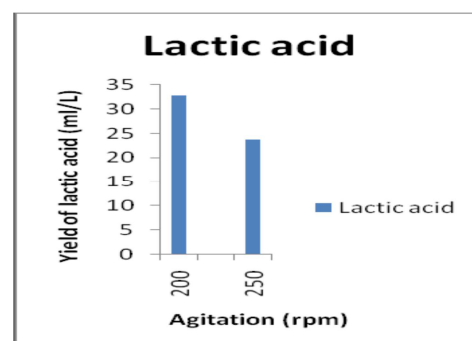
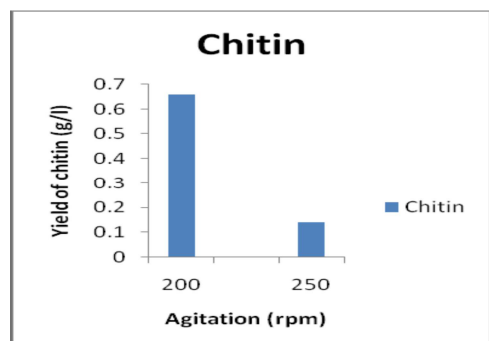


Figure 3 and 4. Comparison of yield of chitin and lactic acid at different pH from *Rhizopus oryzae* NCIM 1009



Figure 5. IR spectrum of commercial chitin

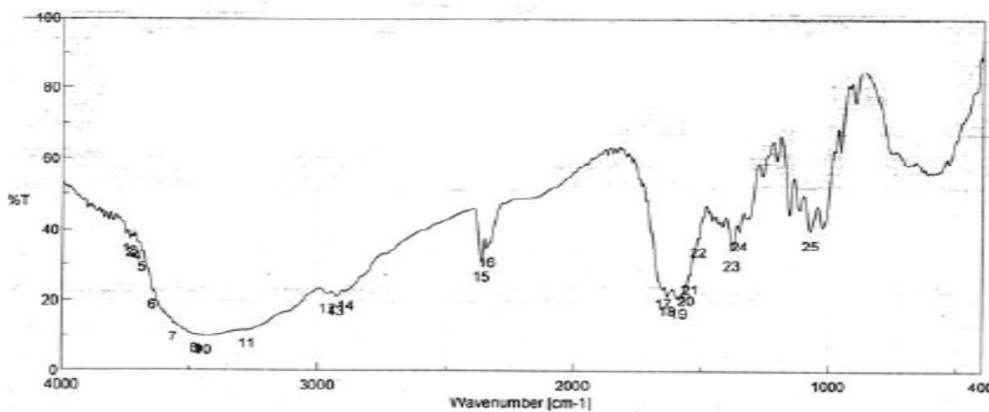


Figure 6. IR spectrum of isolated chitin

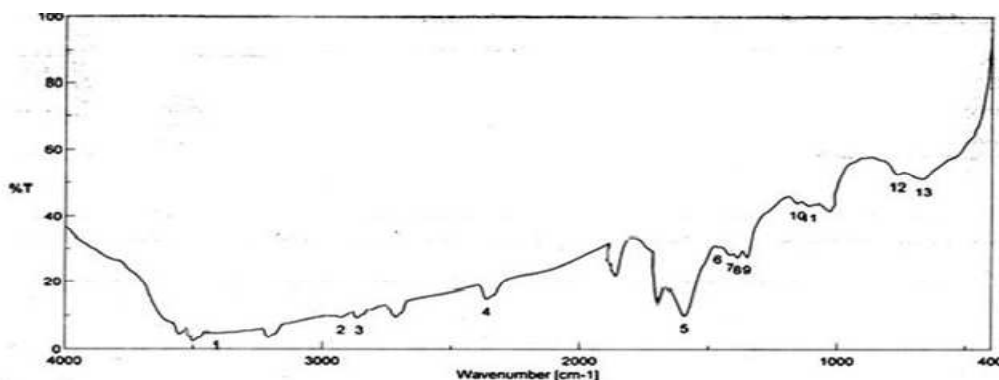


Figure 7. IR spectrum of commercial lactic acid

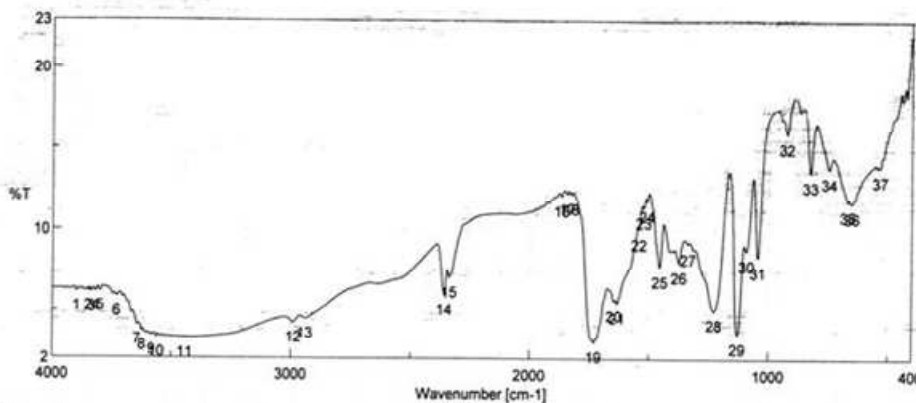
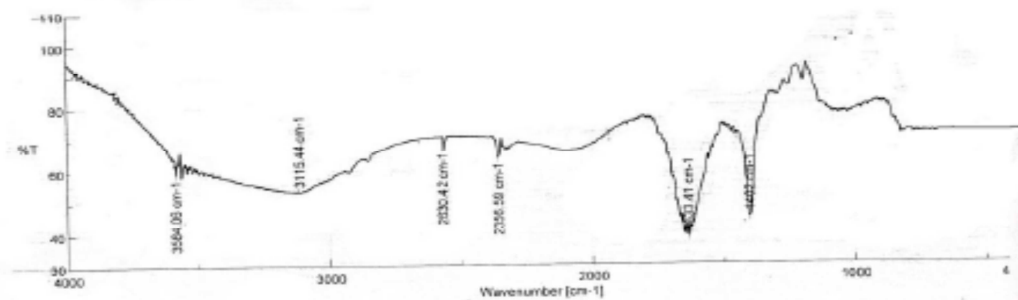


Figure 8. IR spectrum of isolated lactic acid from *Rhizopus oryzae* NCIM 1009

CONCLUSION

Based on the results, it may be concluded that the lactic acid and chitin can be extracted simultaneously and economically from *Rhizopus oryzae* NCIM1009. This work further suggested that lactic acid and chitin production can be optimized further by altering the morphology of mycelia and may genetically engineered in near future.

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