

SCREENING OF NOVEL SUBSTRATES FOR LACTIC ACID PRODUCTION BY *RHIZOPUS ORYZAE*

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ABSTRACT

Lactic acid is a product that finds several applications in food, cosmetic, pharmaceutical and chemical industries. The main objective of this work was the development of a bioprocess to produce lactic acid using dry grass, coconut husk, sugarcane waste and wood chips as substrate. Among the fungal strains, *Rhizopus* species was selected for fermentation, due to its potential for the production of biodegradable and biocompatible polylactate polymers. Fermentation was conducted without need for supplementary other minerals. Lactic acid can be produced from renewable materials using various fungal species of the *Rhizopus* genus, as it demands low nutrient requirements, easy and fast growth and valuable fermentation by-product—fungal biomass. The objective of the experiment is to select a suitable cheap, economic, easily available substrate for production of lactic acid in crystal form by examining its % acid production and xylose utilization on per day basis of fermentation.

Key words : Lactic acid, xylose, *Rhizopus Oryzae*, agro-wastes

INTRODUCTION

Lactic acid, the popular name of 2-hydroxypropanoic acid is the most widely occurring carboxylic acid in the food, pharmaceutical, cosmetics and chemical industries. Its production is currently attracting a great deal of research and development. A review published in 1995 stated that 85% of lactic acid in the USA was used in food and food-related applications, and an emerging application was its use for production of biodegradable and biocompatible polylactate polymers, which provided an environmental friendly alternative to biodegradable plastics derived from petrochemical materials (Datta. R. et al. 1995).

The production of lactic acid can be performed either by chemical synthesis or fermentation. By the

chemical route, the racemic mixture D/L is usually produced, while optically purer isomers (D or L) can be obtained by fermentation when the appropriate microorganism is selected. Lactic acid exists naturally in two optical isomers: D(-)-lactic acid and L(+)-lactic acid. Since elevated levels of the d-isomer are harmful to humans, L(+)-lactic acid is the preferred isomer for food-related and pharmaceutical industries. Renewable resources including sugars, starch and lignocellulose are abundant substrates for fermentative production. The lactic acid global demand is expected to be around 200,000 metric tons per year by the end of 2011, mainly as a result of the growing market for PLA (poly-lactic acid). PLA is a bio-degradable polymer of lactic acid produced from renewable resources, which can replace various

petrochemical industry based polymers in applications ranging from packaging to fibres. The possible sources of carbohydrates for lactic acid fermentation include xylose from sugarcane or beet, dry grass, coconut husk, wood, corn starch, etc from molasses and whey. Pure sugars are generally costly, but facilitate the downstream processing. Alternative substrates such as molasses are cheap, but the high amount of impurities can affect the downstream processing. The main objective of this work was the development of a bioprocess to produce L(+)-lactic acid using above mentioned sources as substrate, concurrently processing an industrial waste and producing a valuable material. Fungal *Rhizopus* species have attracted a great interest, and have been recognized as suitable candidates for lactic acid production. Unlike the LAB, lactic acid producing *Rhizopus* strains generate L-lactic acid as a sole isomer of lactic acid (Soccol. C.R. et al. 1994a; Soccol. C.R. et al. 1994b; Yin. P.M. et al. 1997; Yu. R.C. et al. 1989). The production of L-lactic acid using a surface culture of *Rhizopus* was reported in 1911 (Bulut. S. et al. 2004). An efficient submerged fermentation using fungal species for the production of L-lactic acid was first reported in 1936 (Ehrlich. F., 1911; Lockwood. L.B. et al. 1936). However, an increased research interest has been given to lactic acid fermentation by fungal species in recent decades (Maas, 2006).

Substrate for lactic acid production

A large number of carbohydrate materials have been used, tested or proposed for the manufacture of lactic acid by fermentation. It is useful to compare feedstock on the basis of the following desirable qualities:

1. Low cost.
2. Low levels of contaminants.
3. Fast fermentation rate.
4. High lactic acid yields.
5. Little or no by-product formation.
6. Ability to be fermented with little or no pretreatment.
7. Year-round availability.

Renewable raw materials, including molasses, starch (corn starch, wheat starch, potato starch) and lignocellulose (corn cobs and woody materials) can be used as a substrate for fermentation of lactic acid.

Previous studies on lactic acid production by *Rhizopus* species mainly used glucose as a substrate. The effect of different carbohydrates on L-lactic acid production was investigated by Yin et al. 1997 and Bulut et al. 2004. In many cases, glucose was the preferred carbon source for L-lactic acid production by *Rhizopus* species, followed by starch material. However, as cheap and widely existing materials, dry grass, coconut husk, sugarcane waste and wood were recognized as cost effective carbon sources for lactic acid production. This is due to that the substrate cost is one of the major operational costs, representing 30–40% of total production costs.

MATERIALS AND METHODS

1. Agro-Waste Substrates

A total of 4 different types of agro-wastes viz. dry grass, sugarcane waste, coconut husk, and wood chips; were screened for the amount of xylose content by preparing their hydrolysate and used for lactic acid production by *Rhizopus oryzae*.

2. Preparation Of Hydrolysates:

2.1. Steam Explosion

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

2.2. 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 (Pumiput et al. 2008).

2.3. pH Adjustment

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 (Pumiput et al. 2008).

3. Fermentation (batch culture):

3.1. Microbial strain

The pure culture of *Rhizopus oryzae* NCIM 1299 were grown on solid synthetic Sabouraud's dextrose medium (HIMEDIA). The cultures were then adapted to xylose containing synthetic medium. The composition of synthetic medium used is as follows: Xylose 30g/L, yeast extract 10g/L, peptone 20g/L, K₂HPO₄ 0.5g/L, KH₂PO₄ 0.5g/L, MgSO₄.7H₂O 0.05g/L, (NH₄)₂SO₄ 2g/l, pH 5.0. Xylose was added aseptically after sterilizing separately at 121° C (Shreenath et al. 1986).

3.2. Inoculum preparation

The scraped growth was subcultured in liquid synthetic media containing xylose & different test hydrolysates separately. The inoculum media containing hydrolysate were prepared in 250 mL capacity conical flasks by adding components of synthetic medium (excluding xylose) in 100 mL of each hydrolysate instead of distilled water. The xylose content of hydrolysate supplemented for the carbon source. These media were kept for incubation at ambient temperature on rotary shaker at 120 rpm

for 3 days. These were used as inocula in further studies.

3.3. Media and fermentation conditions

To screen for lactic acid production from agro-waste hydrolysates, 5% of *R. oryzae* inoculum was added to 3L of fermentation media in 4L capacity chemostat bioreactor and incubated at ambient temperature at 120rpm for 4 days. The fermentation medium was similar to the inoculum medium for each test hydrolysate. The substrate (xylose) consumption and also product (lactic acid) formation was examined on daily basis.

RESULTS AND DISCUSSION

1. Screening of different raw materials for xylose content

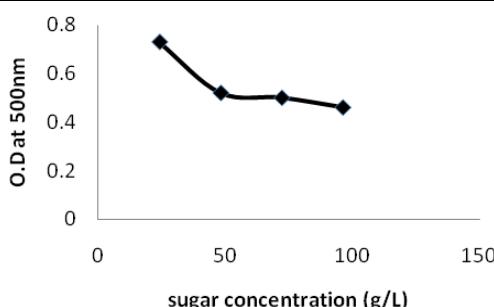
A total of 4 biomass hydrolysates were screened for their xylose content. The acid, conc. HCl at concentration of 2% was observed to give good xylose yield from biomass after hydrolysis.

Table.1: Xylose content in raw material

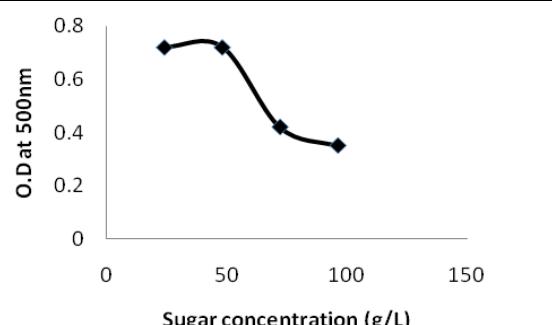
Substrate	Xylose content (gm/L)
Coconut	0.62
Wood chips	0.80
Sugarcane	0.86
Grass	0.92

2. Substrate utilization assay

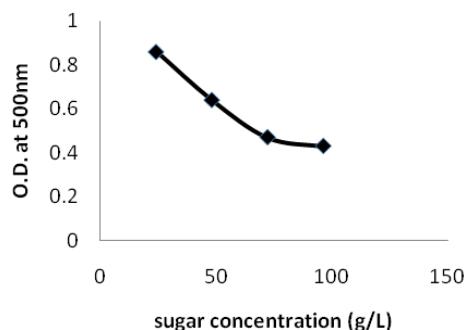
This experiment was performed to analyze the comparative substrate utilization efficiency by *R. oryzae* for product formation. The substrate utilization assay was carried for a period of four days.



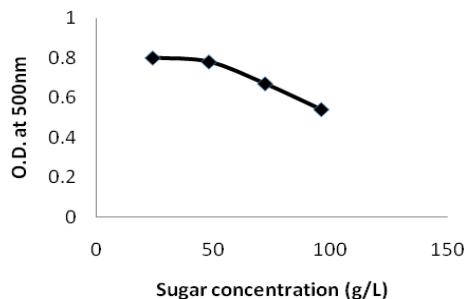
Graph.2a: Substrate utilization assay for sugarcane



Graph.2b: Substrate utilization assay for grass



Graph.2c: Substrate utilization assay for coconut



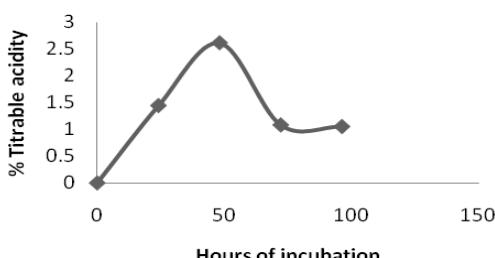
Graph.2d: Substrate utilization assay for wood

3. Lactic acid production

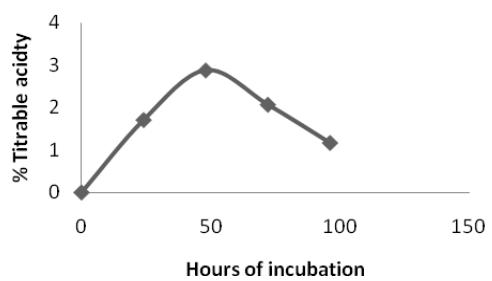
3.1 Titrable acidity

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. The titrable acidity of grass medium was observed to be the highest

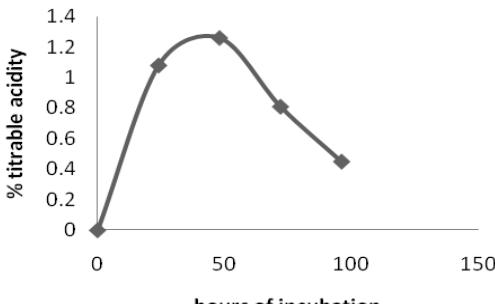
(2.88%), whereas that of coconut husk (1.26%) was observed to be the least among the four substrates used. However, the acidity of the medium was observed to be the highest after 48hrs of incubation, for all the substrates.



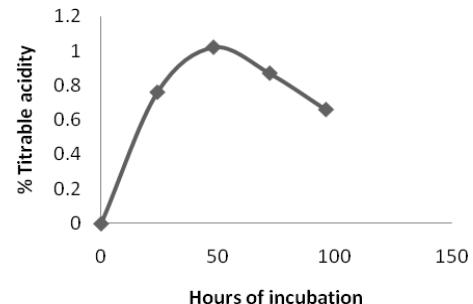
Graph.3.1a: Titrable acidity for sugarcane



Graph.3.1b: Titrable acidity for grass



Graph.3.1c: Titrable acidity for coconut



Graph.3.1d: Titrable acidity for wood

3.2 Lactic acid downstream processing

Lactic acid was purified and the crystals were obtained from the fermentation medium after completion of 48hrs of incubation, by the method described by Sodeck, et. al (1981). The crystals obtained were then used for confirmatory test for lactic acid estimation.

3.3 Lactic acid estimation

Table.2: Concentration of lactic acid

Substrate	Lactic acid concentration (g/L)
Grass	70
Sugarcane	54
Wood	42
Coconut	34

Such high concentrations of lactic acid as compared to the initial sugar content of the substrate was observed because of the process of simultaneous saccharification of substrate sugar and fermentation into lactic acid (Jin et al. 2003 and Zang et al. 2007).

CONCLUSION

This work demonstrated the possibility to produce lactic acid from carbohydrate materials using *Rhizopus* species. The advantages include their amylolytic characteristics, low nutrient requirements, low-cost downstream process for separation of biomass, valuable fungal biomass which can be used in biosorption processes for purification of contaminated effluents, for fungal chitosan production and as additive in animal feeds. Many renewable resources including refined sugars, molasses, raw starch materials and even lignocellulose can be converted to lactic acid using fungal strains of *Rhizopus* genus. However, most

previous investigations revealed that low lactic acid production and productivity is the main limitation associated with the *Rhizopus* species.

Future prospects in this study might include optimization of process parameters, such as pH and oxygen supply, other nutrients, control of morphology which may result in improving performance of *Rhizopus* species in lactic acid production. Molecular genetics and bioinformatics approach to get a better understanding of enzymatic pathways and metabolism, and of genetic regulation involved in lactic acid production using *Rhizopus* species, and consequently for enhancing production yield and productivity.

REFERENCES

1. Barnett. A.J.G. The colorimetric determination of Lactic acid in silage. Biochem J. 1951; Sep 49(4):527-529.
2. Bulut. S., Elibol. M., Ozer. D., Effect of different carbon sources on l(+)- lactic acid production by *Rhizopus oryzae*. Biochem. Eng. J. 2004; 21:33–37.
3. Datta R., Tsai S.P., Bonsignor. P., Moon. S., Frank. J. Technological and economic potential of poly(lactic acid) and lactic acid

derivatives. FEMS Microbiol. Rev. 1995; 16:221–231.

4. Ehrlich. F., Formation of fumaric acid by means of molds. Ber. Dtsch. Chem. Ges. 1911 44:3737–3742.
5. Jin. B., Huang. L.P., Lant. P., *Rhizopus arrhizus*-a producer for simultaneous saccharification and fermentation of starch waste materials to l(+)-lactic acid. Biotechnol. Lett. 2003; 25:1983–1987.
6. Lockwood. L.B., Ward. G.E., May. O.E., The physiology of *Rhizopus oryzae*. J. Agric. Res. 1936; 53:849–857.
7. Maas, R.H.W., Bakker, R.R., Eggink, G. and Weusthuis, R. A. Lactic acid production from xylose by the fungus *Rhizopus oryzae*. Applied Microbiology and Biotechnology 2006; Vol.37(5):861- 868.
8. Pumiput. P, Chuntranuluck. S, Kitpreechavanich. V, Punsuron. V and Vaithanomsat. P. Production process of hydrolysate from Steam Explosion of oil Palm Trunk for Xylitol Fermentation. Kasetsart J. (Nat.Sci) 2008; 42:73-78.
9. Soccot. C.R., Marin. B., Raimbault. M., Lebeault. J.M., Potential of solid state fermentation for production of l(+)-lactic acid by *Rhizopus oryzae*. Appl. Microbiol. Biotechnol. 1994a; 41:286–290.
10. Soccot. C.R., Stonoga V.L., Raimbault. M., Production of l-lactic acid by *Rhizopus* species. World J. Microbiol. Biotechnol. 1994; 10:433–435.
11. Sodeck, G., Modi, J., Kominek, J. and Salzbrunn, G. Production of citric acid according to the submerged fermentation. Process Biochem. 1981; 16(6),9-11.
12. Sreenath, H.K, Chapman, T.W. & Jeffries, T.W. Ethanol production from D-xylose in batch fermentation with *Candida shehatae*: process variables. Applied Microbiology and Biotechnology 1986; 24:294-299.
13. Yin. P.M., Nishina. N., Kosakai. Y., Yahiro. K., Park. Y., Okabe. M., Enhanced production of l(+)-lactic acid from corn starch in a culture of *Rhizopus oryzae* using an air-lift bioreactor. J. Ferment. Bioeng. 1997; 84:249–253.
14. Yu. R.C., Hang. Y.D., Kinetics of direct fermentation of agricultural commodities to l(+) lactic acid by *Rhizopus oryzae*. Biotechnol.Lett. 1989; 11:597–600.
15. Zhang, Y.Z., Jin, B. and Kelly, J.M. Production of lactic acid from renewable materials by *Rhizopus* fungi. Biochem. Engineering J. 2007; 35:251-263.