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Agricultural wastes as substrates and immobilization of spores to increase lactic acid production by *Rhizopus oryzae* ATCC 4858

Ezzat, S.M.[1]; Mansour, M.A.[2]; Fatama, G.K. Othman[1]

1-B*otany Department, Faculty of Science, Zagazig University.*

2-F*ood Hygiene Department, Faculty of Veterinary Medicine, Zagazig University.*

Correspondence to: Fatama G.K. Othman

Mail: Chemist.fatma19@gmail.com

Abstract

The present work explored L-lactic acid (LA) produced from agro-wastes by spores of *Rhizopus oryzae* ATCC 4858 by native hydrolysis prospective of the present work organism. **Immobilization of spores on** agar-agar, carboxymethyl cellulose sodium salt (Na-CMC), artificial fiber sponge, loofa sponge or maize stalks pulp was also investigated. Rice straw gave maximum amount of LA by the fungus. Descending sequence of agricultural wastes according to LA amounts by the fungus is as follows: rice straw > pomegranate peel > orange fruits peel > bagasse > eggplant fruits peel > soya bean meal > wheat bran. Highest amount of lactic acid was produced using spores carried on synthetic sponge than loaf cubic pieces then CMC pellets then pellets of agar-agar and finally cubic pieces of corn stalk pith hemicelluloses. TTA values were the least at agar-agar pellets and maximum at cubic pieces. Final pH value (more acidic) was detected in case of synthetic sponge as a carrier, while the highest was detected on pellets of CMC.

Key words

***Rhizopus oryzae* ATCC 4858, CBS 327.47, DSM 853, lactic acid, agricultural wastes, immobilization,** CMC pellets**, total titratable acidity.**

Introduction

LA is considered as one of the most important component, it was known in 1780 as milk constituent by Scheele, where in 1789, Lavoisier was the first who named this milk component as 'acide lactique'. Meanwhile, Pasteur discovered it as a fermentation product by microbes **(Benninga, 1990).** LA is used in nourishment, brews, self-attention products, therapeutic and poly lactic acid (PLA), a well-known recyclable material **(VickRoy, 1985; Sodergard and Stolt, 2002).** World demand for LA is around 0.3-0.4 million tons per year and is anticipated to be enlarged rapidly in the close imminent **(Nattrass and Higson, 2011).** According to Worldwide Opinion Investigation (California, USA), the universal shop of lactic acid was 714.2 kilo tons in 2013 and 1,960.1 kilo tons in 2020 **(Grand View Research, 2014).**

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The financial price of diverse kinds of remaining produced from agro-industries is mostly below the price of assemblage and reclamation for reuse, which is consequently rejected and cause unfavorable ecological possessions. Though, operative application of waste could be measured as treasured if suitable bioremediation involvements are used for the improvement of new goods, which are ecological safe and cost-effective. The agrowastes discarded has been perceived to be rich in fermentable sugars, which are simply used by the microorganisms and consequently renewed into important constituents **(Ali and Zulkali, 2011).** Lactic acid is an example of such prized materials produced on agrowastes substrates.

The immobilization of microorganisms has ordinarily been used for industrial fermentation to advance the income of the required manufactured goods. In normal deferment culture systems, immobilized entire cells have the virtues of evading wash-out of cells at an extraordinary dilution rate, higher cell concentration in the apparatus and easy separation of cells from the system or the product containing solution **(Frusaki and Seki, 1992).** Hence, the cells have been immobilized by returns of adsorption on polymer supports, by implanting with standard polymers like alginate gels and synthetic polymers **(Tamada et al., 1992).** Many investigators have tried to use immobilization methods for L (+)-lactic acid production with *R. oryzae*. The sting methods using soft gels such as Ca-alginate have mostly been employed in these studies **(Hang et al., 1989).** In gel-entrapping methods, the restriction of oxygen supply because of diffusional opposition might decline the fermentation rate and/or L (+)-lactic acid transformation efficacy **(Dong et al., 1996).** The difficulties, related to filamentous fungal fermentations can be overwhelmed with cell immobilization on support polymer background. In this work, the cells were immobilized by physical entrapment in the open pore network of reticulated loofa and synthetic sponge as well as maize stalk pulp 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).Also, spores are entrapped into pellets of agar and Na - CMC.**

Materials and methods

Microorganism

***Rhizopus oryzae* ATCC 4858, CBS 327.47, DSM 853 was kindly purchased from Egyptian Microbial Culture Collection (EMCC), Microbial Resource Centre (Cairo Mircen).**

Culture medium

ATCC medium 366 – Potato dextrose agar (PDA) was used for maintaining and growing of the fungus throughout this work.

pH:

It was measured using pH meter.

Effect of various agricultural by-products (agro wastes):

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To study the effect of using different agricultural by-products (agro-wastes) as substrates on the growth and lactic acid amounts by ***Rhizopus oryzae*** **ATCC,** 18 different wastes were rice straw chips, corn flour , sugarcane bagasse, corn stalks, rice bran, and wheat bran, sugarcane bagasse , fish meal , corn meal ,dried banana peel, corn stalks , soybean meal , wheat hay, egg plants fruits pericarp peels, pea peels ,orange peels, pomegranate peel , pomegranate pulp , broad bean (fruit pericarp)and cotton stalks. Sugarcane bagasse was obtained from local shops of sugarcane fresh juice at Zagazig, Sharkia Governorate. Corn stalks and rice husks were obtained from some local fields. Rice bran and wheat bran was obtained from the local market at Zagazig, Sharkia Governorate. These materials were oven-dried at 60ºC then milled to 0.2 to 0.5 mm particle size. The waste materials were added in a concentration of 10% and moistened with carbon source free basal broth , initial pH was adjusted to 6.0.The flasks were autoclaved and incubated at 30° C for seven days at 150 rpm in an electric shaking incubator. At the end of incubation period, the cultures were filtered in Whatman filter papers No.1 and final pH, TTA and lactic acid values were determined.

Growth conditions, inoculum preparation and immobilization carriers:

Fungal spores from 5-day old cultures of ***Rhizopus oryzae*** **ATCC 4858** were harvested separately by flooding of the slants with sterile water and gently scrapping off the spores with a sterile glass rod. The spore's concentration was adjusted to 2.0×10^6 spores/ml using haemocytometer and applied for immobilization. Five different carriers were tested as immobilizing carriers. These carriers were agar-agar, carboxymethyl cellulose sodium salt (Na-CMC), artificial fiber sponge, loofa sponge and maize stalks pulp.

Immobilization using Na-CMC carrier:

The method of preparation of inactive Na-CMC immobilized fungal beads was adopted according to **Wang *et al.* (2008)**.100 ml of the CMC solution (2.5 %, w/v) was mixed with 3 ml of conidia suspension (2.0×10^6 spores/ml) of ***Rhizopus oryzae*** **ATCC 4858** until homogeneous. The mixture was injected drop wise to filter-sterilized $FeCl_3$ solution (0.1 mol/l) using an injector forming beads (3-5 mm, diameter). The beads were cured in the $FeCl_3$ solution for 1h to enhance their mechanical stabilities. The immobilized beads (50 in number) were collected, rinsed with sterile distilled water twice and transferred to 250 ml Erlenmeyer flasks containing 50 ml of the PDA broth (pH 6.0). Cultures were allowed to grow at 30°C for 7 days on a rotary shaker at 150 rpm.

Immobilization using agar-agar carrier:

Fungal conidia were immobilized by entrapping into agar-agar (bacteriological grade) carrier, according to the method **of Banerjee *et al*. (1982)** with slight modification. 3.0 g agar was added to 100 ml distilled water and sterilized then cooled to 45°C. Aliquot 3 ml of the freshly prepared conidia suspension (2.0×10^6 spores/ml) of ***Rhizopus oryzae*** **ATCC 4858** was added to the molten agar and shaken thoroughly. This spores agar mixture was then cast into bead shape by injecting it into ice-cold mixture of toluene: chloroform (3:1, v: v), whereby fine beads of

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diameters ranging from 3 to 5 mm were obtained. The beads (50 in number) were then washed repeatedly with phosphate buffer (pH 6.5), air dried and subjected to experimental studies of lactic acid production, as mentioned earlier.

Immobilization using sponge:

Three types of sponge (artificial fiber sponge, loofa sponge and maize stalk pulp) were used for spore's immobilization. The technique was carried out according to **Iqbal and Edyvean (2007**). Artificial fiber sponge and loofa sponge had an average density 0f 0.213 and 0.0312 g/cm^3 with a porosity of 63 and 84 %, respectively. They were cut into discs of 2.0×1.5 (diameter), 2.5 mm (thick), soaked in boiling water for 30 min, thoroughly washed under running water and rinsed for 24 h in distilled water at three exchanges. The sponge discs (7 in number) were then air dried, sterilized and transferred to 250 ml Erlenmeyer flasks containing 50 ml broth medium (pH 6.0).

Estimation of lactic acid

The colorimetric technique of Barker and Summerson **(Gordon and Ralph, 1970)** for estimation of total lactic acid was adapted for use in this study. The method employs the oxidation of both D (-)-and L (+)-lactic acids to acetaldehyde and succeeding formation of a color complex with p-hydroxydiphenyl.

Total titratable acidity (TTA) (AOAC, 1980):

The fungal cultures were filtered through filter papers (What man No.1).Ten ml of the culture filtrate were titrated against 0.1 N NaOH using phenolphthalein as an indicator. End point is the alkali first drop that gives permanent pink color.

Results and discussion:

Agricultural wastes:

Many renewable materials can be used as a carbon source for production of lactic acid by *Rhizopus* spp. These materials include refined sugars (glucose, sucrose, etc.), starch materials, whey, molasses, and other carbohydrate-rich materials such as lignocellulose. The impact of different carbon sources on L-(+)- lactic acid production by Rhizopus arrhizus has been investigated by **Bulut *et al*. (2004).** In this work, different agricultural wastes were air dried and grinded to fine powder. Wastes were added to the basal broth instead of carbon source in a concentration of 10%. Biological production of lactic acid can use cheap raw materials, such as whey, molasses, starch, beet- and cane-sugar and other carbohydrate-rich materials **(Khalaf, 2001 and Martak *et al*., 2003).** The obtained results are present in table 1. It is clear from the results that rice straw gave maximum amount of lactic acid by ***Rhizopus oryzae* ATCC 4858**. The descending sequence of agricultural wastes according to lactic acid production by ***Rhizopus oryzae* ATCC 4858** is as follows: rice straw > pomegranate peel > orange fruits peel > bagasse > egg plant fruits peel > soya bean meal > wheat bran.

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Table 1: Effect of different agriculture wastes on lactic acid .total titratable acidity and final pH values by *Rhizopus oryzae* ATCC 4858

Agriculture wastes	Final pH value	TTA (ml 0.1 N NaOH/10 ml filtrate)	Lactic acid (mg/g byproduct)
Rice straw	5.58	0.26	36.05
Corn flour	5.54	0.3	5.2
Rice bran	5.51	0.56	2.72
Fish meal	5.46	0.53	4.58
Corn meal	5.66	0.8	5.36
Banana peel	5.62	0.53	4.74
Corn stalks	5.80	0.4	3.15
Soybean meal	6.59	0.7	21.06
Bagasse	5.69	0.33	26.69
Wheat bran	5.03	0.43	19.99
Wheat hay	3.64	0.3	9.93
Egg plants peels	4.81	0.43	25.46
Pea peels	5.72	0.8	3.78
Orange peels	4.11	0.56	26.87
Pomegranate peel	4.4	0.53	30.46
Pomegranate pulp	4.03	0.6	9.38
Broad bean (Fruit pericarp)	5.00	0.3	9.84
Cotton stalks	5.81	0.36	9.34

The rest of agricultural wastes gave between 2.72 to 9.84 mg/ml culture medium. It is interesting to mention that The descending sequence of agricultural wastes according to lactic acid production by ***Rhizopus oryzae* ATCC 4858** is as follows: rice straw > pomegranate peel > orange fruits peel > bagasse > egg plant fruits peel > soya bean meal > wheat bran changed the final pH of soybean culture to 6.59, i.e. it reduced acidity, but the more acidic final pH value was observed on wheat hay (pH 3.64). The lowest TTA values was detected on rice straw, while the highest one was detected on corn meal and pea fruits kernels peel.

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Table 2: Effect of different spore's carriers on final pH value & total titrtatable acidity and lactic acid of *Rhizopus oryzae* ATCC 4858. (Immobilization)

Carriers	Final pH value	TTA (ml 0.1 N NaOH/10 ml filtrate)	lactic acid (mg/1 ml culture filtrate)
CMC	4.78	3.3	20.22
Loaf	4.63	4.1	21.73
Agar-agar	4.82	3	13.70
Sponge	3.82	3.9	32.14
Corn wood	4.41	5	9.20

Immobilization made separate liquid medium from the cells much easier **(Nedovic and Willaert, 2004)** and helped numerous recycles of fungal cells for long-term lactic acid production. Lactic acid (LA) fermentation by free and polyurethane (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).**

Different spores carriers were used in this study, 1cm cubic pieces of loaf, synthetic sponge and corn pulp hemicelluloses as well as pellets of Na- CMC and agar-agar were tested as spores carriers as shown in table 2. Highest amount of lactic acid was produced using spores carried on synthetic sponge than loaf cubic pieces then Na- CMC pellets then pellets of agar-agar and finally cubic pieces of corn stalk pith hemicelluloses. TTA values were the least at agar-agar pellets and maximum at cubic pieces. Final pH value (more acidic) was detected in case of synthetic sponge as a carrier, while the highest was detected on pellets of Na-CMC.

It was noticed that loofa and sponge gave more amounts of LA than agar and Na-CMC pellets this may be due to the limitation of oxygen supply because of diffusional resistance might decrease the fermentation rate and/or L(+)- lactic acid transformation efficiency in gel-entrapping methods. The problems, associated with filamentous fungal fermentations can be overcome with cell immobilization on support polymer matrix **(Dong *et al.*, 1996).**

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