

## The Effect of Some Parameters on the Production of L(+) Lactic Acid Using Wheat Wastewater by *Rhizopus oryzae* NRRL-395

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### ABSTRACT

The aim of the study was to investigate some major parameters on production lactic acid using wheat wastewater by *Rhizopus oryzae* NRRL-395. The parameters, which may play an effective role in production, were determined. For developing and producing pure L(+)-Lactic acid of the filamentous fungus *Rhizopus oryzae* NRRL-395, rich medium with wheat wastewater and glucose as carbon source were used in the study. The effects of carbon sources concentration, pH, agitation (shaking speed) and spores concentration on lactic acid production were examined. After fermentation process, the highest values of lactic acid obtained from wheat wastewater and glucose were analyzed using high-performance liquid chromatography with ultraviolet detectors (HPLC-UV). The maximum L (+) Lactic acid production in shake flasks was investigated at pH 6,  $1.0 \times 10^6$  spores/mL, 150 rpm, 30 °C at 8 days. The maximum lactic acid content for wheat wastewater and glucose were obtained at a concentration of 100% (5.638 g/L) and 150 g/L (5.042 g/L). The maximum lactic acid amount of 5.568 g/L was obtained at pH value of 6 for wheat wastewater. However, the maximum lactic acid amount of 2.463 g/L was obtained at pH value of 6 for glucose. The maximum lactic acid values for wheat wastewater and glucose were obtained 5.603 g/L and 2.483 g/L at 150 rpm speed, respectively. The maximum lactic acid values for wheat wastewater and glucose were obtained 5.804 g/L and 5.646 g/L at the  $1.0 \times 10^6$  spores/mL respectively.

### Research Article

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## Atık Bulgur Suyu Kullanılarak *Rhizopus oryzae* NRRL-395 ile Üretilen Laktik Asite Bazı Parametrelerin Etkisi

### ÖZET

Bu çalışmanın amacı, *Rhizopus oryzae* NRRL-395 tarafından bulgur atık suyu kullanılarak üretilen laktik asit üretimine bazı önemli parametrelerin etkisi denenmiştir. Bu amaç ile filamentli bir yapıya sahip olan *Rhizopus oryzae* fungusunun gelişimi ve laktik asit üretimi için bulgur atık suyu ve glukoz gibi karbon kaynakları bu çalışmada kullanılmıştır. Karbon kaynaklarının konsantrasyonunun, pH'larının, çalkalama hızlarının ve spor konsantrasyonlarının üretim üzerine olan etkileri incelenmiştir. Fermentasyon işleminden sonra, bulgur atık suyu ve glukozdan elde edilen yüksek laktik asit değerleri yüksek performanslı sıvı kromatografisi (HPLC-UV) kullanılarak analiz edilmiştir. Çalkalamalı sistemde en yüksek laktik asit değerleri için pH 6,  $1.0 \times 10^6$  spor/mL konsantrasyonunda, 150 rpm hızında ve 30 °C de 8 gün olarak araştırılmıştır. Bulgur atık suyu için maksimum laktik asit miktarı %100 konsantrasyonda (5.638 g/L) ve glukoz için 150 g/L konsantrasyonda (5.042 g/L) olarak elde edilmiştir. Bulgur atık suyu ve glukoz için maksimum laktik asit oranları sırası ile pH 6'da 5.568 ve 2.463 g/L, 150 rpm hızında 5.603 ve 2.483 g/L ve  $1.0 \times 10^6$

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spor/mL konsantrasyonunda ise 5.804 ve 5.646 g/L olarak elde edilmiştir.

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## INTRODUCTION

Wheat is a very important carbohydrate source located at the base of the food pyramid. Wheat wastewater is obtained after the wheat cleaning and boiling process. Besides, the production increases every year, it is concluded that wheat wastewater can be used for lactic acid production and other products in the future (Göçeri, 2013; Anonim, 2020).

Lactic acid is used for a long time to protect human's foods (Davidson et al., 1995). It was firstly found in the sour milk by Scheele in 1780 (Benninga, 1990). In order to produce lactic acid by biotechnological methods, the raw material must be accessible and inexpensive. Because polymer producers demand a vast amount of lactic acid at a low cost. Raw materials should have; cheap, fewer contaminants can be produced quickly, high efficiency, a small number of by-products, to be fast fermentation, and no pretreatment will be required and year-round availability (Åkerberg and Zacchi, 2000). Some low-cost raw materials, which contain carbohydrates, starch, cellulose, wheat wastewater, whey, and

molasses, is used for lactic acid production (Göçeri, 2013; Hofvendahl, 2000). The wheat used for producing lactic acid (Åkerberg, 1998, 2000; Hofvendahl, 1997; Oh et al., 2005). Lactic acid was used as the starting material of polylactic acid (PLA), which has recently been used in the production of biodegradable plastics (Vert et al., 1992). Lactic acid was obtained using both lactic acid bacteria (Bibal et al., 1991; VickRoy et al., 1982) and *Rhizopus oryzae* NRRL-395 could produce considerable quantity of L-lactic acid and use both wheat wastewater and glucose as carbon sources (Göçeri, 2013). Lactic acid-producing fungi, such as *Rhizopus oryzae*, lately gain attention. The important benefit of applying the fungi over the bacteria is that the economic costs, owing to the use of raw and waste substances as well as no need for particular nutrients (Datta et al., 1995; Hofvendahl et al., 1999; Khalaf, 2001). According to the production capacity of lactic acid, microorganisms are divided into two groups as bacteria and fungi. Some organisms producing lactic acid in recent years are given Table 1 (Wee et al., 2006).

Table 1. Biotechnological production of lactic acid of some microorganisms

### *Cizelge 1. Biyoteknolojik olarak laktik asit üreten bazı mikroorganizmalar*

Microorganism (Mikroorganizma)	Lactic acid (g/L) (Laktik asit) (g/L)	Yield (g/g) (Ürün) (g/g)	Productivity g/(L-h) (Verimlilik) g/(L-h)
<i>Rhizopus oryzae</i> ATCC 52311	83.0	0.88	2.6
<i>Rhizopus oryzae</i> NRRL 395	104.6	0.87	1.8
<i>Enterococcus faecalis</i> RKY1	144.0	0.96	5.1
<i>Lactobacillus rhamnosus</i> ATCC 10863	67.0	0.84	2.5
<i>Lactobacillus helveticus</i> ATCC 15009	65.5	0.66	2.7
<i>Lactobacillus bulgaricus</i> NRRL B-548	38.7	0.90	3.5
<i>Lactobacillus casei</i> NRRL B-441	82.0	0.91	5.6
<i>Lactobacillus plantarum</i> ATCC 21028	41.0	0.97	1.0
<i>Lactobacillus pentosus</i> ATCC 8041	21.8	0.77	0.8
<i>Lactobacillus amylophilus</i> GV6	76.2	0.70	0.8
<i>Lactobacillus delbrueckii</i> NCIMB 8130	90.0	0.97	3.8
<i>Lactococcus lactis ssp. lactis</i> IFO 12007	90.0	0.76	1.6

Lactic acid could be produced by humans, microorganisms, plants and animals. In terms of nutritional point, the L(+) form of lactic acid is mainly demanding for the food and industry; as known, the human body is merely designed to absorb this form and just produce L-lactate dehydrogenases (LDH) (Soccol, 1992). Lactic acid is a valuable organic acid commonly used for the food and chemical industry as a preservative, acidulent, flavoring and solvent. Besides, some lactic acid salts are used in the formulation of pharmaceutical products (Kascak et al., 1996; Vick Roy, 1985). Lactic acid can be goods chemical for the

production of lactate, propylene glycol and oxide, acrylic acid, propanoic acid acetaldehyde and dilactide (Vardarajan et al., 1999). According to the US FDA (Food and Drug Administration); It has been reported that the L (+) form of lactic acid can be used as a food additive but form D (-) will be harm human metabolism (Datta, 1995). Production of lactic acid by *Rhizopus oryzae* appears to be an applicable option that could increase in minimum condition both liquid and solid medium (Soccol et al., 1994). This experiment was achieved to identify the best conditions for the production of L(+) Lactic acid by *Rhizopus oryzae*

NRRL-395

## MATERIALS and METHODS

### Microorganism and Inoculums

The fungus used in the study was provided from the İzmir Ege University culture collection laboratory. The fungus was grown on potato dextrose agar (PDA) and transferred to fresh slants every four months and stored at 4 °C for regular sub-culturing. Inoculums preparation; spore cultures provided by suspending spores from 7 days old culture slants of *Rhizopus oryzae*. Spores were grown on PDA slants in 250 mL flask at 30 °C with sterilized distilled water containing 0.2% Tween 80 and filtered with sterile cotton. Spore concentration calculated using by hemocytometer. Erlenmeyer flask culture was inoculated with the latest concentration of  $1.0 \times 10^5$  spores/mL.

### Medium and Cultivation

The study was conducted using a 250 mL Erlenmeyer in shaking culture and wheat wastewater and glucose were used as the carbon sources. Culture medium containing; (10%, 25%, 50%, 75% and 100%) wheat wastewater, and (50, 100, 150, 200, 250 g/L) glucose, 2 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.65 g  $\text{KH}_2\text{PO}_4$ , 0.25 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.05 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ . The medium pH was set 6.0, autoclaved at 121 °C for 15 min than waited at room temperature, inoculated with  $1.0 \times 10^5$  spores/mL, and incubated for 8 days at 30 °C on a rotary shaker at 150 rpm/min. In order to stop the decrease in pH 60% sterilized  $\text{CaCO}_3$  was added to every flask following 24 hours of cultivation.

### Analytical Methods

The fermentation medium was heated for 10 min at 70 °C to dissolve precipitated calcium lactate. The pH of the medium calculated by a pH meter (Thermo-Scientific Orion). The samples were centrifuged for 12 min at 4000 rpm (Sigma-3-16P). The samples were filtered to remove solids and precipitated (pore size of 0.45 µm). The samples were analyzed by high-performance liquid chromatography (HPLC-UV detector) using a Bio-Rad Aminex HPX-87H, 300 mm x 7.8 mm column with 0.008 N sulphuric acid ( $\text{H}_2\text{SO}_4$ ) as the mobile phase at 35 °C and a flow rate of 0.6 ml/dk (Ott et al., 2001; Karaoğul et al., 2016).

## RESULTS and DISCUSSION

Along with the present study, wheat wastewater which is a significant waste of food industry is evaluated for production of lactic acid which is used as the raw material of polylactic acid (PLA) production, which is a biopolymer which can be degraded spontaneously and rapidly in nature. Those studies are also great interests in prevention of the environmental pollution. In this context, many microorganisms were

documented to possess lactic acid production capacities in the previous reports (Zhou et al., 2006). Some of these organisms produce L-(+) form of lactic acid and some form D-(-). Microorganisms such as *Rhizopus oryzae* have few limited aliments demand also could use starch feedstocks (Narayanan et al., 2004).

### The Effect of Wheat wastewater and Glucose Concentration

As it is known, wheat wastewater is a lignocellulosic waste, and glucose is a source of carbon that can be easily digested by many microorganisms. These two carbon sources are being used in the fermentation process (Göçeri, 2013). The results concerned with the effects of wheat wastewater and glucose concentration on the production of lactic acid with *R. oryzae* fungus are collectively represented in Table 2. Herein, lactic acid yield increased as substrate concentration increased as a result of fermentation. The highest amount of lactic acid obtained in the experiments was obtained from 100% wheat wastewater concentration. An increase in the amount of lactic acid was observed with the increase in glucose concentration until 150 g/L. The maximum lactic acid yield was obtained at 150 g/L glucose concentration (Table 2). After the 150 g/L the decrease was attributed to the inhibitory effect dependent on increase in glucose concentration in media and subsequently adversely affects the production by microorganisms. In another study concentration, a decrease in production was observed.

Table 2. The effect of concentration on production

#### Çizelge 2. Konsantrasyonun üretime etkisi

Concentration (Konsantrasyon)	Lactic acid (g/L) (Laktik asit) (g/L)	Amount (%) (Miktar) (%)
Wheat wastewater (%) (Bulgur atık suyu) (%)		
10	0.056±0.008 <sup>a</sup>	2.51
25	1.016±0.009	16.23
50	2.424±0.009	19.40
75	3.005±0.064	19.74
100	5.638±0.013	22.09
Glucose(g/L)(Glikoz)(g/L)		
50	2.393±0.006	14.45
100	3.365±0.009	20.26
150	5.042±0.012	24.17
200	2.315±0.008	14.95
250	2.035±0.009	13.47

<sup>a</sup>Values expressed are means ± S.D. of three parallel measurements

Concentration of the glucose, sucrose, molasses, carob, and wheat bran effect on lactic acid production; the best yield was acquired from the glucose concentration of 150 g/L. Glucose has not been used totally in the present glucose concentration medium and nearly 40-50% unused in the fermentation process (Bulut, 2004).

### Effect of pH

The effect of pH was evaluated by the previous pH and controlled pH throughout the fermentation process.

Production of lactic acid related to the pH cause medium pH strongly effects a lot of enzymatic processes (AbdulRauf, 2010). The medium pH was set at 4, 6 and 8 during the cultivation. The maximum lactic acid amount of 5.568 g/L was obtained at pH value of 6 for wheat wastewater (100 mL). The results are presented in Table 3. However, the maximum lactic acid amount of 2.463 g/L was obtained at pH value of 6 for glucose (50 g/L). Thus; pH 6 was selected as optimum pH for the experiments. pH control method was tested in the experiment by adding NaOH solution at 4 an hour-time period while the cultivation. The pH of close to 6 could be intended to obtain a hopeful microbial success for lactic acid production (Hofvendahl et al., 2000; Iyer and Lee., 1999). In researching the effect of different pH values on production of lactic acid with *R. oryzae*; 0.71 g-g, 0.68 g-g, 0.62 g-g, 0.71 g-g, 0.68 g-g and 0.71 g-g products were obtained at pH 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0, respectively (Dominquez and Vazquez, 1999).

Table 3. Effect of pH on production  
*Çizelge 3. pH'nin üretime etkisi*

pH	Lactic acid (g/L) ( <i>Laktik asit</i> ) (g/L)	
	Wheat wastewater ( <i>Bulgur atık suyu</i> )	Glucose ( <i>Glikoz</i> )
4	4.936±0.013 <sup>a</sup>	1.485±0.001
6	5.568±0.01	2.463±0.019
8	5.485±0.009	1.804±0.001

<sup>a</sup>Values expressed are means ± S.D. of three parallel measurements

### Effect of Agitation (shaking) Speed

In the study conducted to determine the agitation speed, experiments were carried out at 100, 150 and 200 rpm for carbon sources. It was determined that the best yield for wheat wastewater (100 mL) and glucose (50 g/L) were obtained at 150 rpm. The result represented in Table 4. It was chosen as the optimum shaking rate for wheat wastewater because of the fact that there was much difference between 150 rpm and 100-200 rpm. By the way most of the literature studies were performed at 150 rpm (Tanyıldızı et al., 2012). As the agitation rate increases, the amount of oxygen in the fermentation medium increases and this increase causes the microorganism to use more oxygen and produce lactic acid and some organic acids in the medium. As we know that, the lactic acid is merely produced under the anaerobic condition. Despite that lactic acid production is related to the dissolved oxygen in medium. Another reason could be the amount of lactic acid determined by the total biomass in the medium, which interrelating directly with the dissolved oxygen in the medium. In addition, the fungal have mycelia like cotton form which could reduce the oxygen and nutrition mass and finally affected the amount of lactic acid. According to these results some organic acid may influence the decrease

in the amount of lactic acid (Liu et al., 2005; Tkacz and Lange, 2004; Göçeri, 2013).

Table 4. Effect of agitation speed on production  
*Çizelge 4. Çalkalama hızının üretime etkisi*

Agitation (rpm) ( <i>Çalkalama</i> )(rpm)	Lactic acid (g/L) ( <i>Laktik asit</i> ) (g/L)	
	Wheat wastewater ( <i>Bulgur atık suyu</i> )	Glucose ( <i>Glikoz</i> )
100	2.727±0.009 <sup>a</sup>	2.141±0.011
150	5.603±0.009	2.463±0.009
200	2.937±0.019	1.612±0.012

<sup>a</sup>Values expressed are means ±S.D. of three parallel measurements

### Effect of Spore Concentration

In order to determine the effect of spore concentration on lactic acid production by *Rhizopus oryzae*, different spore concentrations including 1.0x10<sup>4</sup>, 1.0x10<sup>5</sup>, 1.0x10<sup>6</sup> to 1.0x10<sup>7</sup> spores/mL were injected into wheat wastewater (100 mL) and glucose (50 g/L) media (Table 5). As a result of the production, it was determined that the best yield values for wheat wastewater and glucose were obtained at 1.0x10<sup>6</sup> spores/mL. Inoculation above 10<sup>6</sup> spore/mL concentration caused a decrease in the amount of lactic acid because the spore concentration increased. Increasing concentration of spores led to starts competition among microorganisms and affected the rapid depletion of carbon sources in the media and the decrease in the amount of lactic acid (Göçeri, 2013). *Rhizopus oryzae* NRRL-395 in their work in the bioreactor using cornstarch to obtain lactic acid with different rates of the effect of vaccination on the production of the spores examined. Spores concentrations were 2x10<sup>3</sup>, 2x10<sup>4</sup>, 2x10<sup>5</sup>, 2x10<sup>6</sup> and 2x10<sup>7</sup> spores/mL respectively. The highest lactic acid production was obtained from 2x10<sup>6</sup> spores/mL (Yin et al., 1997). Much increase inoculum rate effected in a decrease in production because increasing the inoculums rate caused extreme density of spores that decrease the production (Abood, 2017).

Table 5. Effect of spore concentration on production  
*Çizelge 5. Spor konsantrasyonunun üretime etkisi*

Spore Concentration ( <i>Spor Konsantrasyonu</i> )	Lactic acid (g/L) ( <i>Laktik asit</i> ) (g/L)	
	Wheat wastewater ( <i>Bulgur atık suyu</i> )	Glucose ( <i>Glikoz</i> )
1.0x10 <sup>4</sup>	2.352±0.012 <sup>a</sup>	1.411±0.008
1.0x10 <sup>5</sup>	5.603±0.011	2.254±0.014
1.0x10 <sup>6</sup>	5.804±0.004	5.646±0.008
1.0x10 <sup>7</sup>	2.677±0.011	2.608±0.009

<sup>a</sup> Values expressed are means± S.D. of three parallel measurements

### CONCLUSION

In this study, *Rhizopus oryzae* mediated lactic acid production was examined using wheat wastewater

which is discharged to the environment as a waste of wheat production factories. Overall, the best yield and efficiency values of lactic acid were obtained from wheat wastewater using *Rhizopus oryzae* NRRL-395 fungus. This microorganism was used in all subsequent stages. Based on these results, the production of lactic acid using wheat wastewater by *Rhizopus oryzae* at pH 6, 150 rpm and  $1.0 \times 10^6$  spores/mL was the appropriate condition for production. In this experiment shows that wheat wastewater is better than glucose for producing lactic acid. Besides, 60% percent of the water used as one liter in the wheat production phase is wheat wastewater, of which 5.804 g/L lactic acid was obtained. Accurately, wheat wastewater is appropriate for the lactic acid fermentation and could be used as the unique nutrient for lactic acid production by *Rhizopus oryzae* fungi. Further investigation will be needed to get higher yields, quantities and to optimize condition for the production of L-lactic acid by *R. oryzae*

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#### Statement of Conflict of Interest

Authors have declared no conflict of interest.

#### Author's Contributions

The contribution of the authors is equal.

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