GIDA (2016) 41 (5): 299-304 *Research* / Araştırma
doi: 10.15237/gida.GD16018

EFFECT OF NaCl AND KCl SALTS ON GROWTH AND LACTIC ACID PRODUCTION OF *RHIZOPUS ORYZAE*

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Received / Geliş Tarihi: 22.01.2016
Received in revised form / Düzeltilerek Geliş Tarihi 01.02.2016
Accepted / Kabul Tarihi 13.02.2016

Abstract

Rhizopus group organisms produce a variety of fermented foods and industrial products including enzymes, organic acids, lipid derivatives, pesticides, herbicides, antibiotics. Some strains of *Rhizopus* are good lactic acid producers, with many advantages over lactic acid producing bacteria. Lactic acid has a wide range of application in food processing industry. In this study, growth and lactic acid production of *Rhizopus oryzae* in response to different NaCl and KCl concentrations in the culture were investigated. The growth and lactic acid production depended on the salt concentration, they decreased as the salt concentration increased. The type of salt did not result in significant differences in terms of growth and lactic acid production.

Key Words: *Rhizopus oryzae*, lactic acid, salt

NaCl VE KCl TUZLARININ *RHIZOPUS ORYZAE*'NIN BÜYÜMESİNE VE LAKTİK ASİT ÜRETİMİNE ETKİSİ

Özet

Rhizopus grubu organizmalar enzimler, organik asitler, lipit türevleri, pestisitler, herbisitler ve antibiyotikleri içeren endüstriyel ürünler ile çeşitli fermente gıdaları da üretirler. *Rhizopus*'un bazı suşları laktik asit üreten bakterilere göre daha avantajlı laktik asit üreticisidirler. Laktik asit gıda işleme endüstrisinde geniş bir uygulama alanına sahiptir. Bu çalışmada *Rhizopus oryzae*'nin besiyerindeki farklı NaCl ve KCl derişimlerine cevaben büyüme ve laktik asit üretimi araştırılmıştır. Büyüme ve laktik asit üretimi tuz derişimine bağlıdır, tuz derişimi arttıkça azalmışlardır. Tuzun türü büyüme ve laktik asit üretimi açısından kayda değer bir farka neden olmamıştır.

Anahtar kelimeler: *Rhizopus oryzae*, laktik asit, tuz

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INTRODUCTION

Fungi are able to synthesize many materials important to food, drug and chemical industries. *Rhizopus group* is important in the production of fermented food products like bongkrek, khaomak, lao-chao, oncom, tape ketan, tapuy, tauco, tempeh (1, 2). Some properties of *Rhizopus* species make them preferred in food production. They can grow in a wide range of temperature, between 25 and 45 °C (3, 4). Moreover they produce a high number of secondary metabolites with sensorial and nutritional interest like β-carotene, ergesterol, lactic acid (5, 6). Also they can produce anti-fungal and anti-bacterial compounds (7). *R. oryzae* is considered GRAS by the U.S FDA. *R. oryzae* does not produce any mycotoxin (8), even though some of *Rhizopus* species have been reported to produce mycotoxins (9). In fact, *Rhizopus* species can be used as a detoxifying agent against food toxins, like Ochratoxin A (10) and on the other hand, to increase the digestibility of certain legumes (11).

R. oryzae was first described in 1936 as a lactic acid producer. Lactic acid is a naturally occuring multifunctional organic acid. It is found in many food products, mainly in fermented food items. It is used in a wide range of food processes from soda to sausages production and in non-food industrial applications because it preserves, enhances flavour or imparts desired acidity (6).

In the literature, there are studies on the effect of oxygen rate, pH of medium, $CaCO_3$ addition, inoculum size, concentration of nitrogen source, agitation speed and aeration rate on the morphology and lactic acid production. Indeed morphology and lactic acid production of *R. oryzae* are interdependent, since the growing mycelia causes highly viscous broth and a dramatic decrease in oxygen and mass transfer rate. Therefore, controlling mycelia morphology is important in order to get higher production rates (12, 13).

In this study, the effect of salt type and concentration on the growth and lactic acid production of *R. oryzae* was investigated. To the best of our knowledge, there are currently no reports in the literature published on the relationship between salt and growth in *R. oryzae*. Throughout the study, macroscopic and microscopic images of mycelia were examined in addition to dry weight determination and lactic acid production to define the growth characteristics and product formation.

MATERIALS AND METHODS

Microorganism and growth conditions

R. oryzae (ATCC 9363) was used in this study. It was sporulated on streaked agar plates containing Potato Dextrose Agar for 4-5 days at 30 °C. After sporulation, the plates were stored at 4 °C until preparation of spore suspension. For fermentation study, agar plates containing sporulated fungus were washed by sterile water to obtain spore suspension. For each inoculation, spore concentration in the suspension was determined by counting the spores on hemocytometer. *R. oryzae* was inoculated into the liquid media composed of (all w/v) 2% glucose, 0.2% $(NH_4)_2SO_4$; 0.065% KH_2PO4; 0.025% $MgSO4_4.7H_2O$ and 0.005% $ZnSO_4.7H_2O$ and then the cultures were incubated in shaker- incubator at 35 °C and 150 rpm.

R. oryzae secretes significant amounts of lactic acid to the growth media, which leads to the decrease in pH down to 2.0–2.3 after 24 h. Hence, 1% $CaCO_3$ was also added to the cultures to neutralize the secreted lactic acid; buffering capacity of $CaCO_3$ is sufficient to keep pH between 6.1–6.5 in the cultures.

In order to examine the effect of salts on the growth of *R. oryzae*, salt ((w/v) 4%; 2%; 1%; 0.5% of NaCl and (w/v) 4%; 2%; 1%; 0.5% of KCl) were added to the growth media.

250 ml-Erlenmeyer flasks containing 100 ml of medium were used. The cultures were inoculated with 10^5 spores/ml. Fermentation duration was six days, glucose was totally consumed in the cultures by then.

Analytical methods

Glucose and lactic acid concentration was determined by HPLC. The HPLC method is based on a normal chromatography procedure, using Phenoemenex (California, USA), Rezex Cal organic acid column column size of which is 300x7.8 mm. and a refractive index detector. Eluent was 5 mM H_2SO_4 flowing at 0.6 ml/min. The volume of injection for both standard solutions and sample extracts was 20 µl.

The morphology was characterized by macro- and microimaging. For macro imaging, a generic digital camera was used. For micro imaging, a digital microscope imager (Celestron, CA, USA), mounted on a light microscope (Bresser, Germany) was used to capture the images.

Dry weight measurements

Mycelia were harvested by filtering the media, washed with distilled water, filtered again, dried in pre-weighed aluminum folia for 24 hours at 90 °C, and finally were weighed to obtain the dry weights.

The student's t-test was used for statistical analysis.

RESULTS AND DISCUSSION

Physiological conditions of media like pH, type of carbon and nitrogen source and their amounts, minerals, size of inoculum are well known to have great influence on morphology of *R. oryzae* (12, 14). Besides these conditions, salt effect on the morphology and growth should be determined. Therefore, a study was conducted which included two types of salts (NaCl and KCl) with different concentrations.

A salt-free control flask and NaCl containing flasks were inoculated and incubated in shaker incubator. As shown in Figure 1a, the macroscopic appearances of the mycelial growth can be defined as non-uniform size pellet in cultures containing 0.5 and 1% NaCl, pellet in culture containing 2% NaCl, uniform size pellet in culture containing 4% NaCl, and congealed mass in the control culture. Biomass dry weights obtained were 65, 50, 26, 8 and 3 mg/L for 0%, 0.5%, 1%, 2% and 4% NaCl containing cultures, respectively (Figure 2).

Tarı *et. al.* (2011) reported the effects of the different concentrations of Mg^{2+} and Zn^{2+} salts on the morphology of *R. oryzae*; small clumps became fluffy pellets with mycelium in response to an increase of Mg^{2+} concentration, changing Zn^{2+} concentrations in growth media did not cause any difference (15). In the literature there are many studies on the physiological responses of yeasts (especially *S. cerevisiae*) to salt stress (16). However, the available researchs on molds focus on the molecular mechanisms and morphological changes in halotolerant/ halophilic/ xerophilic strains (17, 18).

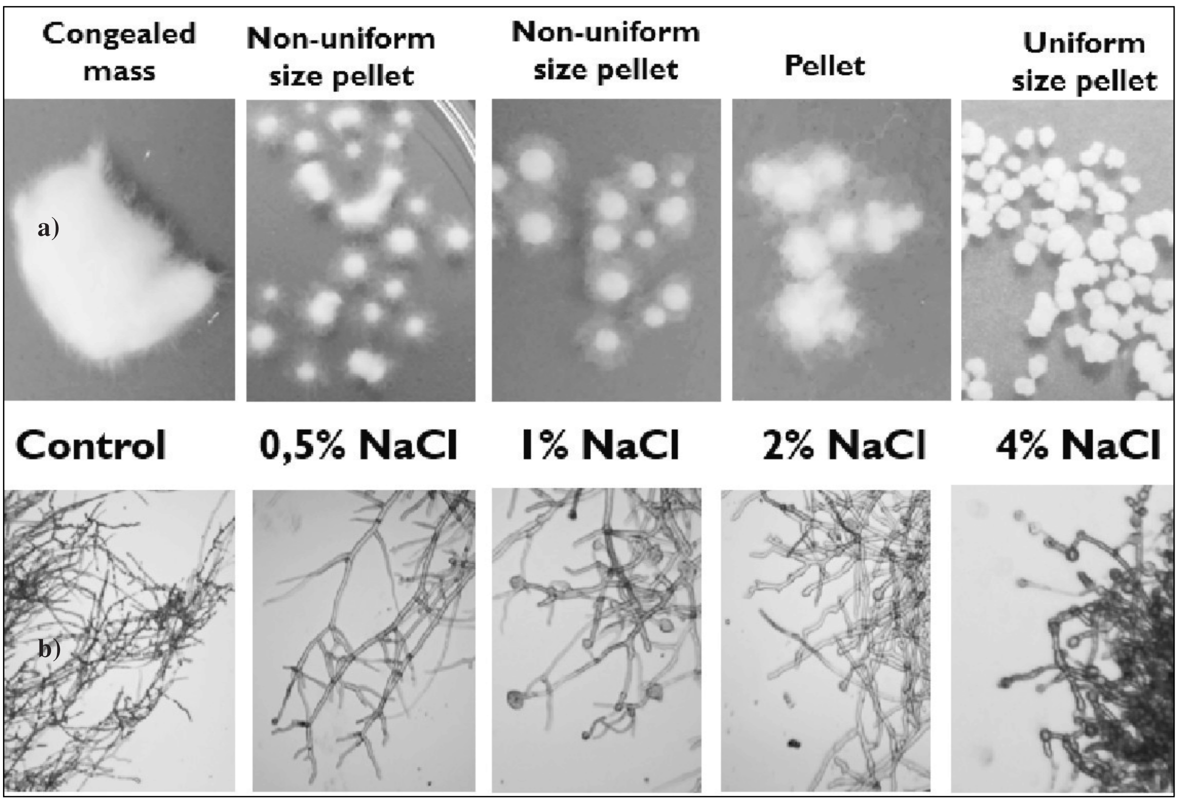

Figure 1. The morphological differences in *R. oryzae* at different NaCl concentrations. a) macroscopic appearances, b) microscopic appearances.

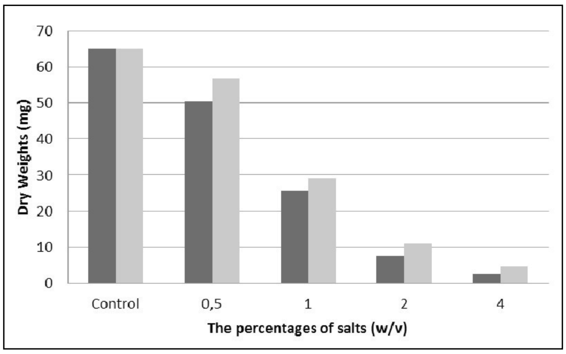

Figure 2. The effect of different NaCl (Black bars) and KCl (Gray bars) percentages on growth of *R. oryzae*.

In the case of KCl containing media, after the inoculation and incubation, the macroscopic appearances of the mycelial growth can be defined as non-uniform size pellet in cultures containing 0.5 and 1% KCl, dispersed in culture containing 2% KCl, pellet in culture containing 4% KCl, and congealed mass in the control culture (Figure 3a). Biomass dry weights obtained were 65, 57, 29, 11 and 5 mg/L for 0%, 0.5%, 1%, 2% and 4% KCl containing cultures, respectively (Figure 2).

The microscopic appearances (i.e. number of tips, hyphal length) were comparable and did not differentiate in both NaCl and KCl containing cultures (Figure 1b and 3b).

According to these results, it can be suggested that the type of the salt used (NaCl/KCl) did not result in significant differences (P>0.05) in the final mycelia dry weights, however the salt concentration affected the biomass dry weights considerably. The effect may be attributed to the increase in the osmotic stress by the increasing salt concentration (19).

After the discovery of *R. oryzae* being a producer of optically pure L(+) lactic acid, many studies were made to understand its lactic acid production mechanism and the conditions affecting the production rate of lactic acid (20-24).

In our study it was found that the lactic acid production depended on the salt concentration, not on the type of the salt (Figure 4). The lactic acid production was in the same range up to 1% salt concentration, after which it decreased as the salt concentration increased, for both salt types studied, most possibly due to the repressive effect of salts on the growth and lactic acid formation.

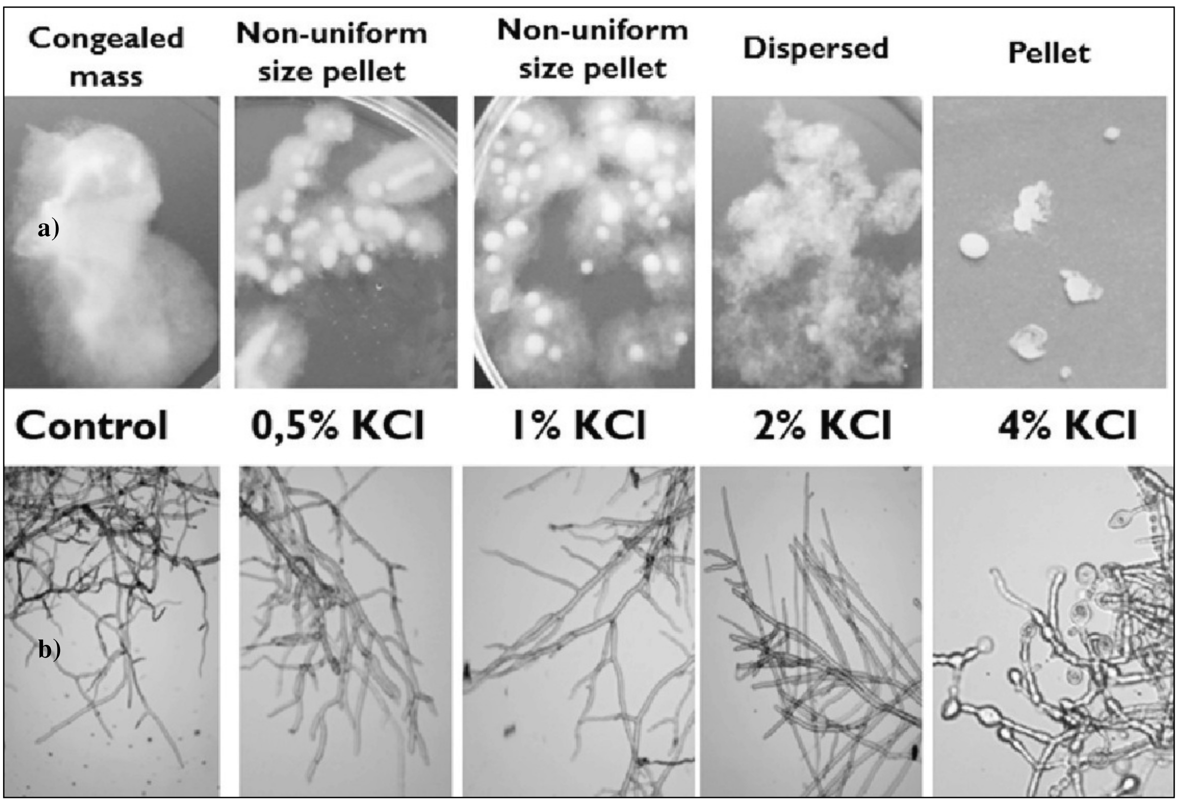

Figure 3. The morphological differences in *R. oryzae* grown at different KCl concentrations. a) macroscopic appearances, b) microscopic appearances.

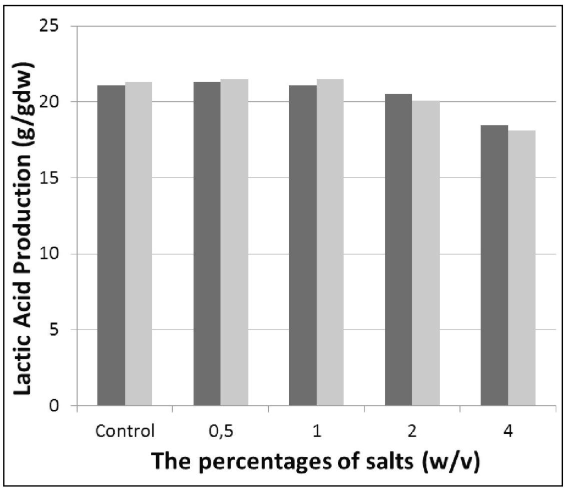

Figure 4. The effect of different NaCl (Black bars) and KCl (Gray bars) concentrations on the lactic acid production by *R. oryzae*.

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