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## Biochemical Role of Yeast Enzymes during Ripening Period in the Egyptian Ras Cheese (Romy)

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### ABSTRACT

These tests consisted of counting, isolation, and identification of the yeasts. The highest value of yeast count in Ras cheese samples was 1095 cfu/g in Fa1 sample which taken from Dakahlia Governorate in fresh samples. The lowest value was 60 cfu/g in Fa2 sample which taken from Kafr El-Sheikh Governorate in Dayer samples. Twenty-five yeast isolates were collecting from the yeasts which grown on Rose Bengal yeast extract sucrose agar medium. The shape of yeast colonies were varied between oval and irregular of colonies, and the appearance of them were glossy and slime. Colonies dimensions were varied between 0.5 and 18 mm. Nine yeasts were identified based on morphological, biochemical, and physiological characteristics in Al-Azhar university represented in the regional center for Mycology & Biotechnology and the results were *Candida parapsilosis*, *C. lusitanae*, *Pichia anomala*, *Hansenula anomala*, *C. pelliculosa*, *C. lusitanae*, *C. famata*, *C. lipolytica* and *Debaryomyces hansenii*. Enzymes which hydrolysis proteins, lipids, and sugars by yeast during the period of ripening were studied. All yeast species were tested for proteolysis and lipolysis on PDA medium. All yeast strains gave a positive result in sugars hydrolysis and produced acids. Most yeast strains did not give gas in the Durham's fermentation tube. Finally, the ability of yeast isolates to produce organic acids were tested. All yeast isolates gave fast results and a high concentration for producing lactic and formic acid. It was in a lower concentration in the case of citric, oxalic, and tartaric acid, but acetic acid production was small and at lowest rate.

### Key words:

Yeast enzymes, lactose fermentation, organic acids production, proteolysis, Ras cheese and Ripening.

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### INTRODUCTION

Cheese ripening is a slow and expensive biological process whose outcome cannot be fully predicted or controlled. During the past 50 years, dairy researchers have tried to decrease the time of cheese ripening and thus reduce the costs of cheese-making by using some ways to accelerate the ripening of cheese without changing the final product such as flavor (El Soda and Awad, 2011). The biochemical process of cheese ripening is hard to study specially in crude soft and smooth and flavor. Two agents of acceptability flavor and texture. Time of storage for changing flavor and texture up to few weeks and three years in soft and very hard cheeses, respectively. During the ripening period chemical and microbiological changes were done, especially in the main component of cheese such as protein, fat and lactose which hydrolysis to less complicated component and secondary products (El-Fadaly et al., 2015a and b). All microorganisms had an important role in Ras cheeses. During manufacture Cheese contamination with bacteria, fungi and yeasts caused the cheese spoiled as well as cheese flavor. Yeasts fermented cheese and gave carbon dioxide and ethanol during manufacture of cheese (Gonçalves et al., 2017). *Geotrichum*

*candidum* and *Debaryomyces hansenii* cultures were used for cheese making form a long time ago and also in recent time and increased year after year (Fröhlich-Wyder et al., 2018). This study was carried out to examine, count and isolate yeasts of Ras cheese. Also, Glycolysis, proteolysis, and lipolysis study in the biochemical properties of Ras cheese yeast strains and the role of them in the ripening of the Egyptian Ras cheese. Finally identified these yeasts according there morphological, physiological, and biochemical properties.

### MATERIAL AND METHODS

#### Ras cheese sample collection:

Samples of Ras cheese (nine) were collected from three different factories, three samples for each of them. These factories located in (Fa 1) (Dakahlia Governorate), (Fa 2) (Kafr El-Sheikh Governorate), (Fa 3) (Damietta Governorate). Three Ages of these samples which were (Fresh) (days from manufacturing), Dayer (middle age sample two or three months old) and old sample (Bayet) (year or over).

**Cultivation media:**

The study were used three cultivation media for counting and isolation the yeasts which were Potato dextrose agar (PDA) (Difco, 2009), Rose Bengal yeast extract sucrose agar (RYS) (Ronald, 2006) and Yeast extract glucose chloramphenicol agar (YG Agar) (Ronald, 2006).

**Yeast and fungi count:**

The technique method which used in this study was pouring plate method, 1 ml of first, second and third dilutions of all cheese samples collected was transferred aseptically in PDA medium (Difco, 2009), and inoculated each of three sterile petri dishes. About 50 ml of PDA medium at approximately 50°C was poured in each sterile petri dishes. Petri dishes were mixed will and left until solidification. All plates were kept in an incubator at 25°C for three days. After this period, colonies of fungi and yeasts in all plates were developed and these colonies were counted and calculated as the yeasts and fungi count (APHA, 1998).

**Yeast isolation:**

YG Agar medium (Ronald, 2006) was used for yeast isolation. Single yeast colonies which a different morphological properties were isolated. Colonies were inoculated at RYS plates for sub culturing to obtain pure isolates yeast. The maintaining of yeast isolates were done on RYS medium slants at 5°C till use (Ronald, 2006).

**Yeast Photographing**

Yeast photographing was done using a compound light microscope and also, a digital eye piece with a magnification 10× was used. The yeasts were photographed.

**Yeast identification**

The identification was done in Al-Azhar University at The Regional center for Mycology & Biotechnology. Yeast identification was based on the colonies characteristics, the microscopic feature of yeast colonies, the growth requirements of yeast cultures, assimilation and fermentation of some sugars and nitrogen (Garnier *et al.*, 2017 and Geronikou *et al.*, 2020).

**Biochemical activities of yeast strains (proteolysis and lipolysis)**

Plates of PDA medium containing casein or oil were inoculated with the chosen yeasts (9 isolates). The incubation period was three days at 25°C, the plates were fill in with HCl with a concentration 10% or CuSO<sub>4</sub> a concentration 10%, respectively. Biochemical activities of yeast strains for each proteolysis and lipolysis were done by measuring the diameter of growth and clear zone for proteolysis process and by recorded the plates which give blue or green color around the colonies for lipolysis process (El-Fadaly *et al.*, 2015a).

**Sugar fermentation:**

Nutrient broth medium containing 5 g of lactose, fructose or sucrose were distributed in test tubes, each tube was containing bromothymol blue and Durham's tubes. Sterilized tubes containing different sugars were inoculated with one loop of each isolated yeasts (9 isolates). After three days of keeping the cultures in the incubator at 25°C, the production of acid in the yeast cultures were studied (the color change from blue to yellow) and the gas production in Durham's tubes were recorded (Difco, 1998).

**Organic acids determination:**

Oxalic, tartaric, citric, lactic formic and acetic acids were determined in chemistry laboratory of faculty of agriculture Damietta university using ferric chloride solution test (Klein, 2013).

**RESULTS AND DISCUSSION****Yeast and fungi count of Ras's cheese samples:**

Table 1 showed that, the count of yeast and fungi in Ras cheese samples. The highest value was 1095 cfu/g in Fa1 sample which taken from Dakahlia Governorate in fresh samples. The lowest value was 60 cfu/g in Fa2 sample which taken from Kafr El-Sheikh Governorate in Dayer samples. Generally, the yeast count of fresh samples was always in the highest level and then decline to the lowest level in the Dayer samples, in the last age (Bayet) the count of yeast increased again until rechecked to a value between Dayer and fresh. These results were lower than that obtained by Al-Gamal *et al.* (2019) who counted the yeast in Ras cheese which collected from different markets from three different Egyptian governorates; Cairo, Giza and Menofia and the results was  $4.5 \pm 1.96 \log \text{CFU/g}$ .

**Table 1: Yeast and fungi counts of Ras cheese samples**

Factories located	Sample ages*	Yeast and fungi count (cfu)/g**
Dakahlia Governorate	Fa 1 fresh	1095
	Fa 1 Dayer	105
	Fa 1 Bayet	605
Kafr El-Sheikh Governorate	Fa 2 fresh	235
	Fa 2 Dayer	60
	Fa 2 Bayet	101
Damietta Governorate	Fa 3 fresh	1375
	Fa 3 Dayer	156
	Fa 3 Bayet	715

\*Three Ages of these samples which were (Fresh) (days from manufacturing), Dayer (middle age sample two or three months old) and old sample (Bayet) (year or over)

\*\*Where: cfu means colony forming unit

**Yeast isolation:**

Twenty-five yeast isolates were collecting from RYS medium. Table 2 showed that the shape of colonies which was 17 of them was oval colonies and the other 8 colonies were irregular. By

studding the appearance of colonies, it was observed that 19 of them were glossy and slime. Colonies dimensions were varied between 0.5 and 18 mm. According to similarity 20 isolates were chosen and used for the further experiments.

**Table 2: The morphological characteristics of yeast colonies isolates**

Isolate code	Colonies shape		Colonies dimensions (mm)		
	Outside look	The appearance	Length	Width	Diameter
1	Oval	glossy and slime	-	-	2:4 mm
2	Oval	glossy and slime	-	-	1:4 mm
3	Oval	None	-	-	0.5:3 mm
4	Oval	glossy and slime	-	-	1:4 mm
5	Oval	None	-	-	0.5:3.5 mm
6	Oval	glossy and slime	-	-	0.5:3mm
7	Oval	glossy and slime	-	-	0.5:2.5 mm
8	Oval	glossy and slime	-	-	2:5 mm
9	Irregular	glossy and slime	18	10	-
10	Irregular	glossy and slime	16	11	-
11	Irregular	glossy and slime	6	5	-
12	Oval	glossy and slime	-	-	1.5:7 mm
13	Oval	None	-	--	1:5 mm
14	Irregular	None	7	6	-
15	Irregular	None	6	5.5	-
16	Oval	glossy and slime	-	-	0.5:6 mm
17	Oval	glossy and slime	-	-	1:6 mm
18	Oval	None	-	-	1:5.5 mm
19	Oval	glossy and slime	-	-	1:8 mm
20	Oval	glossy and slime	-	-	1:3 mm
21	Oval	glossy and slime	-	-	0.5:2 mm
22	Irregular	glossy and slime	6	4	-
23	Irregular	glossy and slime	7.5	6	-
24	Irregular	glossy and slime	5	2	-
25	Oval	glossy and slime	-	-	6

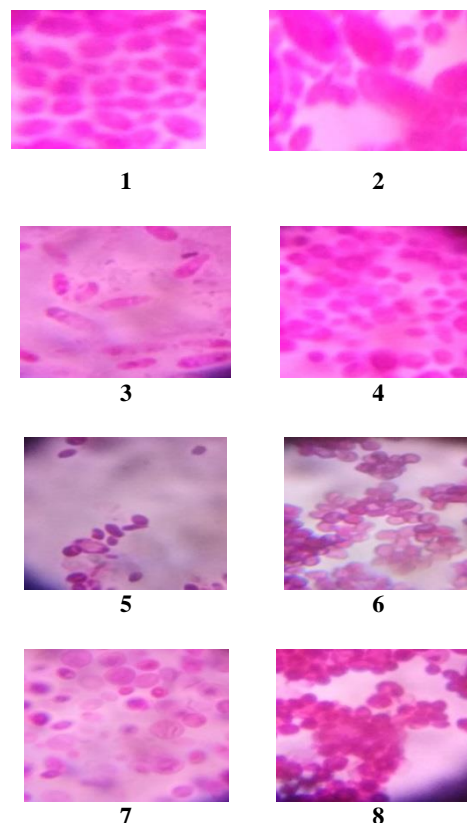
**Yeast Photographing**

Twenty isolates were examined by using a light microscope. The yeasts cells were photographed by digital microscope eye piece. Table 3 and Fig. 1 showed that, 4 isolates were cylindrical cell,

and 16 isolates were oval. The cell dimensions were varied between 1.8 to 19.8 μ.

**Table 3: Yeast Colonies shape and dimensions**

Isolate code	Cell shape	Cell dimensions (μ)		
		Length	Width	Diameter
1	Oval	-	-	4.5
2	Cylindrical	19.8	5.4	-
3	Cylindrical	18	6.3	-
4	Oval	-	-	6.3
5	Oval	-	-	6.3:10.8
6	Oval	-	-	4.5
7	Oval	-	-	4.5
8	Oval	-	-	4.5:9.9
9	Oval	-	-	6.3
10	Oval	-	-	8.1
11	Oval	-	-	8.1
12	Oval	-	-	6.3
13	Oval	-	-	4.5
14	Oval	-	-	6.3
15	Cylindrical	13.5	5.4	-
16	Oval	-	-	9
17	Oval	-	-	7.2
18	Oval	-	-	9:13.5
19	Oval	-	-	6.3
20	Cylindrical	2.7:9.9	1.8:2.7	-





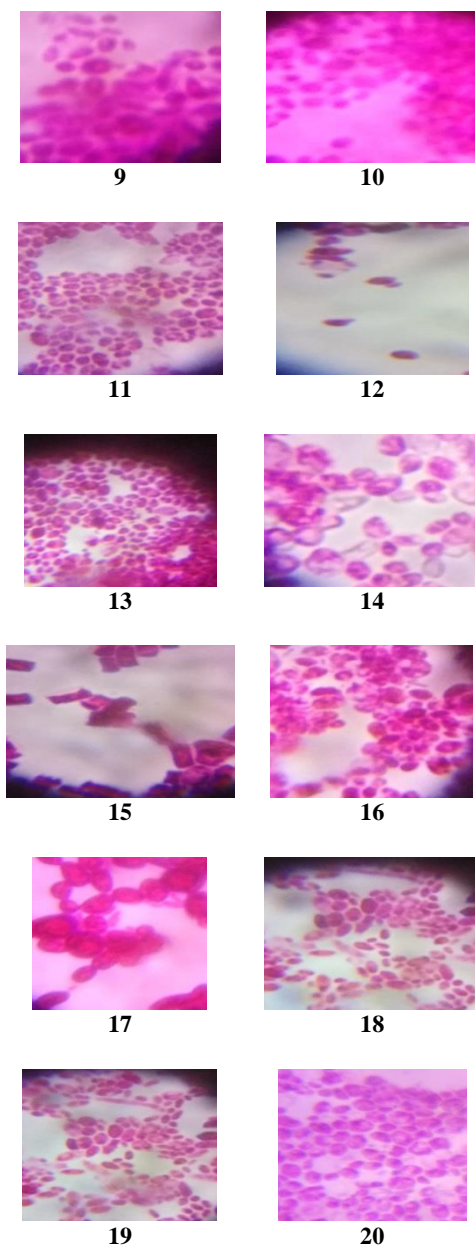


Fig 1. Yeasts isolates Code and cell shape using a light microscope by a digital microscope eye piece with a magnification 400x

**Efficiency of the yeast strains for proteolysis and lipolysis**

All yeast species were tested for proteolysis and lipolysis on PDA medium. It was found that, yeasts had a very good growth and it give a clear zones around the colonies in the case of proteolysis (Table 4). Also these yeasts give green color around the colonies in the case of lipolysis (Table 4). So that, it was observed that these yeasts had both hydrolysis casein and lipid, which indicated that these yeasts had a good role in cheese ripening of protein and lipid. On the same trend, the finding of El-Fadaly *et al.* (2015b) reported that, all tested fungal strains which isolated from Ras

cheese during repining period, showed positive results for casein hydrolysis. The chemical composition of Ras cheese during storage confirmed the fungal enzyme activities, where the fat percentage value increased after salting and during ripening period.

**Table 4: Biochemical tests of different yeast isolates:**

Isolate code	Biochemical tests							
	Sugars						Protein	Fat
	Lactose		Fructose		Sucrose			
	Acid	Gas	Acid	Gas	Acid	Gas		
1	+	+	+	+	+	-	+	+
2	+	-	+	-	+	-	+	+
3	+	-	+	-	+	-	+	+
4	+	+	+	+	+	+	+	+
5	+	-	+	-	+	-	+	+
6	+	-	+	-	+	+	+	+
7	+	-	+	-	+	-	+	+
8	+	-	+	-	+	-	+	+
9	+	-	+	-	+	-	+	+

**Efficiency of the yeast strains for sugar hydrolysis**

Table 4 also showed that, all yeast strains gave a positive in acid but not in gas. Most yeast strains did not give gas in the Durham’s fermentation tube. Similar results were obtained by Kure *et al.* (2001) and Kure and Skaar (2000) Residual lactose is metabolized quickly to lactate during the early stages of ripening at a rate largely determined by temperature and the salt-in-moisture (S/M) levels of the curd (McSweeney and Sousa, 2000 and McSweeney, 2004). The finding of El-Fadaly *et al.* (2015a and b) reported that, thirteen fungal strains which isolated from Ras cheese during repining period, gave negative results in lactose assimilation except *A. nidulans* which produced acid without gas after 24 hr and produced a soluble red pigment after 10 days of incubation at 25°C.

**Identification of yeasts isolated from Ras cheese samples:**

Nine yeasts were identified based on biochemical and morphological properties. These isolates were isolated from the Ras cheese samples and identified after a selective process that was carried out based on the physiological and biochemical characteristics obtained, as well as the external appearance of the different samples. Yeasts were identified In cooperation with Al-Azhar university represented in the regional center for Mycology & Biotechnology. Table 5 showed that, two isolates were obtained from the first factory (F1) and these were defined as *Candida parapsilosis* and *C. lusitanae*. In the second Factory (F2) there were three isolates and there were defined as *Pichia anomala*, *Hansenula anomala* and *C. pelliculosa*. The last Factory (F3), there were four isolates were obtained from it, *C. lusitanae*, *C. famata*, *C. lipolytica* and *Debaryomyces hansenii*. Al-Gamal *et al.* (2019) isolated a yeast from Ras cheese and identified as *C. parapsilosis*. These results were in agreement with Merchán *et al.* (2020) who reported that, the most important yeasts isolated

from cheese were *D. hansenii*, *Galactomyces* spp., *K.marxianus*, *K. lactis*, *Pichia* spp., *Candida* spp. and *Yarrowia lipolytica*.

**Table 5: Identification of some yeasts isolates and its locations.**

No.	Sample code	Locations	Identification
1	(F1) S2D1	Dakahlia Governorate	<i>Candida parapolosis</i>
2	(F1) S3D1	Dakahlia Governorate	<i>Candida lusitanae</i>
3	(F2) S3D2	Kafr El-Sheikh Governorate	<i>Pichia anamola</i>
4	(F2) S2D2	Kafr El-Sheikh Governorate	<i>Hansenula anomala</i>
5	(F2) S3D1	Kafr El-Sheikh Governorate	<i>Candida pelliculosa</i>
6	(F3) S2D2	Damietta Governorate	<i>Candida lusitanae</i>
7	(F3) S2D1	Damietta Governorate	<i>Candida famata</i>
8	(F3) S3D1	Damietta Governorate	<i>Candida lipolytica</i>
9	(F3) S2D3	Damietta Governorate	<i>Debaryomyces hansenii</i>

#### Organic acids production by yeast strains:

Table 6 showed that all yeast isolates gave fast results and a high concentration for producing lactic and formic acid. And it was in a lower concentration in the case of citric, oxalic, and tartaric acid, but acetic acid production was small and at lowest rate. These organic acids were responsible for the odder and taste of Ras cheese. On the other hand, these organic acids have a good role to prevent the growth of harmful fungi and its secretion of aflatoxins. **Awad (2006)** reported that, the fermentation always occurs by the native microflora from the raw milk and the environment. Moreover, Ras cheese is usually stored in moist and uncontrolled hygienic conditions which support the growth of fungi and yeasts. Consequently, the final flavor and texture will be influenced by the actions of all these factors. These founding were matching with **El-Fadaly et al. (2015b)** who published that, the effect of organic acids as antifungal agents on the growth of some fungi which isolated from Ras cheese during repining period such as *A. flavus* and *Rhizopus nigricans*. At the same trend, **Hassan et al. (2012)** found that, acetic acid showed the highest rate inhibition effect on *A. flavus* growth while citric and tartaric acid gave the lowest rate inhibition effect. acetic, propionic, and Formic acids had the highest rate inhibition effect on *A. flavus* growth. Also, these organic acids reduced aflatoxin secretion specially for *Rhizopus nigricans* in the presence of formic acid.

**Table 6: Acid production by yeast strains:**

Yeast strains	lactic acid	citric acid	oxalic acid	tartaric acid	formic acid
<i>Candida parapolosis</i>	++	+	+	+	++
<i>Candida lusitanae</i>	++	+	+	+	++
<i>Pichia anamola</i>	++	+	+	+	++
<i>Hansenula anomala</i>	++	-	+	+	++
<i>Candida pelliculosa</i>	++	+	+	+	++
<i>Candida lusitanae</i>	++	+	-	-	++
<i>Candida famata</i>	++	+	+	+	++
<i>Candida lipolytica</i>	++	+	+	+	++
<i>Debaryomyces hansenii</i>	++	-	-	-	++

(++) Very high concentration, visible and fast result.

(+) High concentration and appears after a while.

(-) The concentration is very low and does not appear until after a long time.

(--) Almost zero concentration.

## CONCLUSION

After conducting various biochemical tests, it was found that, yeasts had a significant role and influence in the flavor and texture of Egyptian Ras cheese during different storage periods specially in the taste and odder.

## FUNDING:

This research was self-funded.

## CONFLICT OF INTEREST:

The authors declare that they have no conflict of interest.

## AUTHORS CONTRIBUTION

Hamad, M. N. F. and El-Kadi, Sh. M. L. developed research proposal, shared manuscript preparation and revision.

Gamal, A. handled the experiment and measurements and shared manuscript preparation.

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## الدور البيوكيميائي لإنزيمات الخميرة خلال فترة التسوية في الجبن الراس المصرية (الرومي)

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### الملخص العربي

جمعت تسع عينات من جبن الراس المصري من مصادر مختلفة وأجريت عليها تحليلات ميكروبيولوجية. تتألف هذه الاختبارات من عد الخمائر وعزلها وتعريفها. كانت أعلى قيمة لعدد الخميرة في عينات جبن الراس كانت ١٠٩٥ مستعمرة / جرام في عينة Fa1 المأخوذة من محافظة الدقهلية في العينات الطازجة. أقل قيمة كانت ٦٠ مستعمرة / جرام في عينة Fa2 المأخوذة من محافظة كفر الشيخ في عينات الداير. جمعت خمسة وعشرون عزلة خميرة من الخمائر التي نمت على اجار مستخلص الخميرة والسكر بوجود صيغة الروزبنغال. كان شكل مستعمرات الخميرة متنوعاً بين المستعمرات البيضاوية وغير المنتظمة، وكان مظهرها لامعاً ولزجاً. تراوحت أبعاد المستعمرات بين ٠.٥ و ١.٨ ملم. تم تعريف تسع خمائر بناءً على الخصائص المورفولوجية والفسولوجية والاختبارات البيوكيميائية في جامعة الأزهر ممثلة في المركز الإقليمي لعلم الفطريات والتكنولوجيا الحيوية وكانت النتائج عبارة *candida parapolosis*, *candida lusitanae*, *pichia anomola*, *Hansenula anomala*, *Candida pelliculosa*, *candida lusitanae*, *candida famata*, *candida lipolytica* & *Debaryomyces hansenii*. تم إجراء اختبارات الدور البيوكيميائية لإنزيمات الخميرة خلال فترة النضج بقدرة عزلات الخميرة على تحليل البروتينات والدهون والسكريات. تم اختبار جميع أنواع الخميرة من أجل تحلل البروتينات وتحلل الدهون على وسط اجار مستخلص البطاطس أعطت جميع سلالات الخميرة نتيجة إيجابية في حالة تخمير السكريات والأحماض المنتجة. معظم سلالات الخميرة لا تعطي الغاز في أنبوب التخمر في دورهام. أخيراً، تم اختبار قدرة عزلات الخميرة على إنتاج الأحماض العضوية. أعطت جميع عزلات الخميرة نتائج سريعة وتركيز عالٍ لإنتاج حمض اللاكتيك والفورميك. وكان بتركيز أقل في حالة حامض الستريك والأكساليك والطرطريك، ولكن إنتاج حمض الأسيتيك كان ضئيلاً وبأدنى معدل.

### الكلمات المفتاحية

إنزيمات الخميرة، تخمير اللاكتوز، إنتاج الأحماض العضوية، تحلل البروتين، جبن الراس، التسوية.



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