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Role of Fungal Enzymes in the Biochemistry of Egyptian Ras Cheese during Ripening Period

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Abstract

Six fungal genera including thirteen species isolated from Ras cheese samples were tested for assimilation of lactose, skim and fat milk hydrolysis. All fungal strains 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. All tested fungal strains showed positive results for protein hydrolysis on modified Czapek agar (MCA) medium by skim milk addition. The presence of casein in the cultural medium caused a strong growth comparing with the growth on control. Changing of growth resulting from milk casein addition was in the highest values in the cultures of *Rhizopus stolonifer*, *A. flavus* and *A. niger* being 87.81, 58.32 and 41.58 mm, respectively and the lowest values were *A. nidulans* and *Geotrichum candidum* being 7.09 and 13.71 mm. All strains gave positive results for lipolysis except *Aspergillus nidulans* on plates containing modified Czapek agar (MCA) medium by milk fat addition. The fungal growth diameter was varied from 9.17 to 30.67 mm in the case of *A. niger* and *A. alliaceus*, respectively. The presence of milk fat in the cultural medium caused a changing of growth in the case of *A. glaucus*; *A. alliaceus* and *A. flavus* being 28.11, 27.42 and 25.19 mm, respectively and *A. niger*, *Rhizopus stolonifer* and *A. flavipes* achieved lowest values being 5.62, 10.80 and 11.82 mm, respectively. Moisture percentage was gradually decreased during storage period (3 months at 15.5˚C) from 31.29% in the first month to 30.25% in the third month. Fat percentage was 24.05% in fresh Ras cheese wheels, which increased to 25.89% after salting period (45 day). During ripening period the values of fat in Ras cheese wheels were 30.53%, 35.98% and 32.38% after the 1st, 2nd and 3rd month, respectively. The highest values of total nitrogen, soluble nitrogen and non-protein nitrogen percentages of Ras cheese wheels were 4.18%, 43.97% and 18.98%, respectively. These values tended to increase with advanced storage.

Keywords

Fungal Enzymes, Glycolysis, Proteolysis, Lipolysis, Egyptian Ras Cheese (Romy), Ripening Rooms

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1. Introduction

Cheese ripening is a very complex biochemical process by which the rubbery or elastic curd is converted into a smooth-bodied and fully flavoured cheese. Flavour and texture are considered as the two main criteria in determining the acceptability of aged cheese. The time that is required to develop characteristic flavour and texture varies from a few weeks for soft cheeses up to three years for very hard varieties. During this period, cheeses attain their own characteristics through a multitude of chemical, microbiological and biochemical changes whereby protein, fat and residual lactose are broken down to primary products which are further degraded to secondary products [1]-[3].

Microorganisms are an essential component of all natural cheese varieties and play important roles during both cheese manufacture and ripening. Cheese is a very involved microbial ecosystem, and a very complex microflora develops in most cheese varieties. Microorganisms, present in cheese throughout ripening, play a significant role in the ripening process, and selection of suitable strains would enable the cheese maker to control or modify flavour development. However, due to the complexity of the flora and the interactions which occur between individual components of it and the cheese environment, strain selection for flavour improvement is not always very obvious. The advent of molecular techniques to study cheese microflora will lead to a major increase in our understanding of this ecosystem and this knowledge will be harnessed to control cheese ripening [3] [4].

During the ripening of cheese, three major biochemical events occur (glycolysis, lipolysis and proteolysis), each of which is involved in flavour formation. The latter is the most important and also the most complex. Glycolysis is the conversion of lactose to lactic acid and is due to the growth of starter bacteria, and the lactate produced gives the freshly made cheese its overall acidic taste. They can also produce diacetyl, acetate and acetaldehyde, which are important compounds in flavour formation in fresh cheeses; diacetyl is also an important flavour compound in hard cheeses. Lipolysis results in hydrolysis of the milk fat and production of glycerol and free fatty acids, many of which, particularly the short-chain ones, have strong characteristic flavour. The fatty acids can be further metabolized to methyl ketones and fat also acts as a solvent for many of the flavour compounds produced in the cheese. The sources of proteinase in cheese are milk itself, chymosin (rennet), starter lactic acid bacteria, nonstarter lactic acid bacteria, and the secondary microorganisms (micrococci, yeasts and moulds). Milk proteinase is plasmin and is significant in cheese when the chymosin is inactivated during cooking of the cheese [1] [5] [6].

Fungi are important in the ripening of a range of cheeses. Mould ripened cheeses are divided into two groups: those ripened due to the presence of *Pencillium roqueforti* which grows and forms blue veins within the cheese, such as Roquefort, Gorgonzola, Stilton and Danish blue, and those ripened with *P. camemberti* which grows on the surface of the cheese, such as Camembert and Brie. Moulds are associated with a range of other cheese varieties also; however, the moulds and their impact on ripening in these cheeses are less well understood [7]. The dry cheeses are placed in natural caves in the production area, where ripening takes place at a nearly constant relative humidity (90% - 95%) and temperature (9˚C - 12˚C). Under these conditions, *Penicillium roqueforti* enters the cheese and develops in the matrix, providing the final product with its characteristic appearance. Microbiologically, this cheese offers a complex habitat in which prokaryotic and eukaryotic populations interact and develop throughout manufacturing and ripening [8]. The moulds have more complex enzymatic systems than bacteria and their enzymes contribute in cheese maturing of the cheese, *i.e*., to proteolysis and lipolysis which are more extensive in these cheeses [9]. Many authors published a lot of papers about the accelerating of cheese ripening including the use of enzymes like proteinases, peptidases, and lipases. These approaches have shown that the addition of free enzymes to milk or cheese led to uncontrolled biochemical reactions [10].

Ras cheese (Romy), the main traditional hard cheese in Egypt, is manufactured in a high proportion under artisan conditions from raw cow's or mixture of cow's and buffalo's milk without using starter cultures and marketed when it has a queried sharp flavour closed to kefalotyic cheese after 3 to 6 months [11] [12]. The fungal growth of the surface of Ras cheese looks like a white felt after a few weeks of storage such as *Geotrichum candidum*, *Aspergillus ochraceus*, *A. alliaceus*, *A. oryzae*, *A. niger*, *A. nidulans*, *Emericella nidulans*, *A. flavus*, *A.*

glaucus, *Penicillium* sp., *Mucor* sp. and *Rhizopus stolonifer* [13] [14]. Also, the surface of the French cheeses is covered by a complex fungal flora containing *Penicillium*, *Mucor*, *Cladosporium*, *Geotrichum*, *Epicoccum* and *Sporotrichum*, while *Penicillium* and *Mucor* have been reported on the surface of the Italian cheese Taleggio and Geotrichum on that of Robiola [7]. Interior or surface mould-ripened cheeses have different appearances and the high biochemical activities of these moulds produce the typical aroma and taste [3] [4]. On the other hand, mould growth on Jarlsberg and Norvegia (the two most commonly produced and consumed semi-hard cheeses in Norway) can periodically be a quality problem. Mould growth can then be observed on the cheese during ripening, during storage at the factory or during retail distribution. The cheeses become spoiled due to the visible mould colonies on the surface and the off-flavours they produce. The mould growth may also represent a health risk because of the possibility of mycotoxin production by some mould species [15].

This work aims to study the role of fungal enzymes in the three major biochemical events: glycolysis, proteolysis and lipolysis during ripening period of Egyptian Ras cheese (Romy).

2. Materials and Methods

2.1. The Fungal Strains

Six fungal genera including thirteen species were isolated and identified in Agric. Microbiology Dept., Fac. of Agric., Damietta University, Damietta, Egypt as following: *Geotrichum candidum*; *Aspergillus ochraceus*; *A. alliaceus*; *A. oryzae*; *A. niger*; *A. nidulans*; *Emericella nidulans*; *A. flavus*; *A. glaucus*; *Penicillium* sp.; *Mucor* sp.; *Rhizopus stolonifer* and *A. flavipes* [13] [14].

2.2. Maintenance of Fungal Strains

The fungal strains were maintained on potato dextrose agar (PDA) medium slants [16] at 5˚C till use. Before use, the fungal strains were subcultured on new slants of PDA and incubated at 25˚C for 5 days.

2.3. Fungal Spores Photographing

Using a microscope (Bio-4o2-B Microtech, made in Japan) and a digital microscope eye piece (640 × 480) (10× WF made in China). The fungal spores were photographed and the concentrations were calculated.

2.4. Spores Suspension of the Tested Fungal Strains

Spores suspensions were prepared as described by Osman [17]. Fungi were grown on PDA slant at 25˚C for 10 days. The spores were harvested in sterilized tap water. 10 ml of sterilized saline solution (0.09% NaCl) was added to the slants and the spores were loosened by gently brushing with a sterile inoculating loop. A vortex mixer (VM-300 power: 220 VAC, 50 Hz, 0.16 A/made in Taiwan-associated with Cannic, Inc., USA) was used for one min. to remove all spores from slant [18]. Spores count was performed in a Hematocytometer slide and the following equation was used for fungal spores count:

$$\text{Spores count/ml} = \text{mean of spores count in 10 squares} \times \text{slid factor} \times \text{dilution ratio.}$$

where slid factor = 2.5×10^6 Hematocytometer slide (model Buerker MOM BUDA pest).

2.5. Lactose Assimilation

Lactose fermentation was done on a medium containing the following composition (g/L): peptone, 5.0; beef extract, 3.0; lactose, 5.0; bromothymol blue, 0.03 ml; and the pH was adjusted to 7.0. Each test tube was supplemented with Durham's fermentation tube. Sterilization was done in Arnold sterilizer at 100˚C for 60 min for three successive days. All tubes were incubated with approximately 1×10^6 spores per tubes. The tubes were incubated in a digital incubator (Switc, MPM Instruments s.r.l., Bernareggio/made in Italy) at 25˚C. The developed color was observed during the incubation period (10 days), the production of acid was recognized by the change in color from blue to yellow and the production of gas was noted in Durham's tubes [19].

2.6. Qualitative Caseinase-Lipase Activity

Casein and milk fat hydrolysis were done on two different cultivation media. The first one was modified potato

dextrose agar (MPDA) medium and the second was modified Czapek agar (MCA) medium [13] [14]. The measurement was carried out using modified potato dextrose agar (MPDA) medium, one ml of sterilized skim milk or milk fat was added to PDA medium. All plates were spot inoculation by tested fungal strains. After incubation at 25˚C, the plates of casein and milk fat were flooded with hydrochloric acid (10%) or cupper sulfate (10%), respectively [20]. The results were recorded by measuring the diameter of growth and clear zone using a digital vernier caliper, Made in Jiangsu China [14]. In case of modified Czapek agar (MCA) medium, the chemical composition (g/L) was K2HPO4, 1; Czapek concentrate, 10 ml and agar agar, 15. The pH was adjusted to 5.2, sterilized by autoclaving at 121˚C for 15 min [21]. A modification was occurred in this medium to be suitable for casein and fat hydrolysis. One ml of sterilized skim milk or fat of milk was added to every Petri dish. Skim milk and fat of milk were separately sterilized in Arnold sterilizer at 100˚C for 60 min for three successive days and this medium was used for testing the isolated fungi for proteolysis and lipolysis, respectively [14].

The chemical composition of Czapek concentrate was as follow (g/100ml): $NaNO_3$, 30; KCl, 5; $MgSO_4 \cdot 7H_2O$, 5 and $FeSO_4 \cdot 7H_2O$, 0.1 Czapek concentrate will keep indefinitely without sterilization. The precipitate of $Fe(OH)_3$ which forms in time can be resuspended by shaking before use [21].

2.7. Ras Cheese Wheels Manufacturing and Its Chemical Analysis

Ras Cheese was manufactured in El-Ghazy laboratory, Elsawalem, Damietta, Egypt. The procedure suggested by Yossef [22] and Abou-Donia [23] for making Ras cheese was adopted. After completely salting process (dry salting for forty-five day in salting rooms), the Ras cheese wheels were stored at about 15˚C for one month in a commercial ripening room, then the Ras cheese wheels were transferred in a digital incubator at 60˚F (15.5˚C) for another two months, thereafter samples were taken after one, two and three months. Ras Cheese was analyzed for fat, soluble nitrogen (SN) and non-protein nitrogen contents (NPN) according to Ling [24].

3. Results and Discussion

3.1. Adjustment of Fungal Spores Count

Spores of all the tested fungal strains were counted by a Hemocytometer slide (**Figure 1**). It was observed that, *Aspergillus nidulans* was the highest value of spores count being 60×10^6 spore/ml, but *Mucor* sp. was the lowest number being 7×10^6 spore/ml (**Table 1**). Spores suspension were prepared to give approximately 1×10^6 spores per ml using the data presented in **Table 1**, by adding the volume of solution A (spore suspension) to the volume of solution B (saline solution) for adjustment the fungal spores count to give a final concentration of 1×10^6 spores/ml using a micropipette (Micro Volume pipettor-Accumax A Made in China), thus the count of spores became fixed in the following experiments.

3.2. Efficiency of the Fungal Strains for Lactose Assimilation (Glycolysis)

Most lactose in milk is lost in whey as lactose or lactate during cheese manufacture. However, low levels of lactose remain in the curd at the end of manufacturing (0.8% - 1.0%). Residual lactose is metabolized quickly to lactate (Glycolysis) during the early stages of ripening at a rate largely determined by temperature and the salt-in-moisture (S/M) levels of the curd [25] [26]. **Figure 2** showed that, all fungal strains gave surface growth, but negative results in lactose assimilation except *A. nidulans* (F6) which produced acid without gas after 24 hr. This observation was explained by two theories, the fungal deacidification and the formation of alcohols. *P. camemberti* metabolises the lactate to CO_2 and H_2O, which results in deacidification of the cheese surface within three weeks.

The outer part of Camembert undergoes considerable modification of texture and the curd, which is firm and brittle at the beginning of ripening, later becomes soft. The surface flora establishes a pH gradient from the surface (basic) to the interior (acidic) due to consumption of lactic acid and NH_3 production. The increase in pH and breakdown of α_{s1}-casein by rennet are responsible for the softening of the curd, which gradually extends towards the center, and is visible in a cross-section of the cheese. The production of methyl ketones by *P. roqueforti* are inhibitory to further mould growth, and may be a factor in preventing excessive mould development in blue veined cheese. The strong reducing conditions in hard cheeses may favour the formation of alcohols from aldehydes. The findings of [1] [4] [7] [14] [27] may explain why the acid formation decreased.

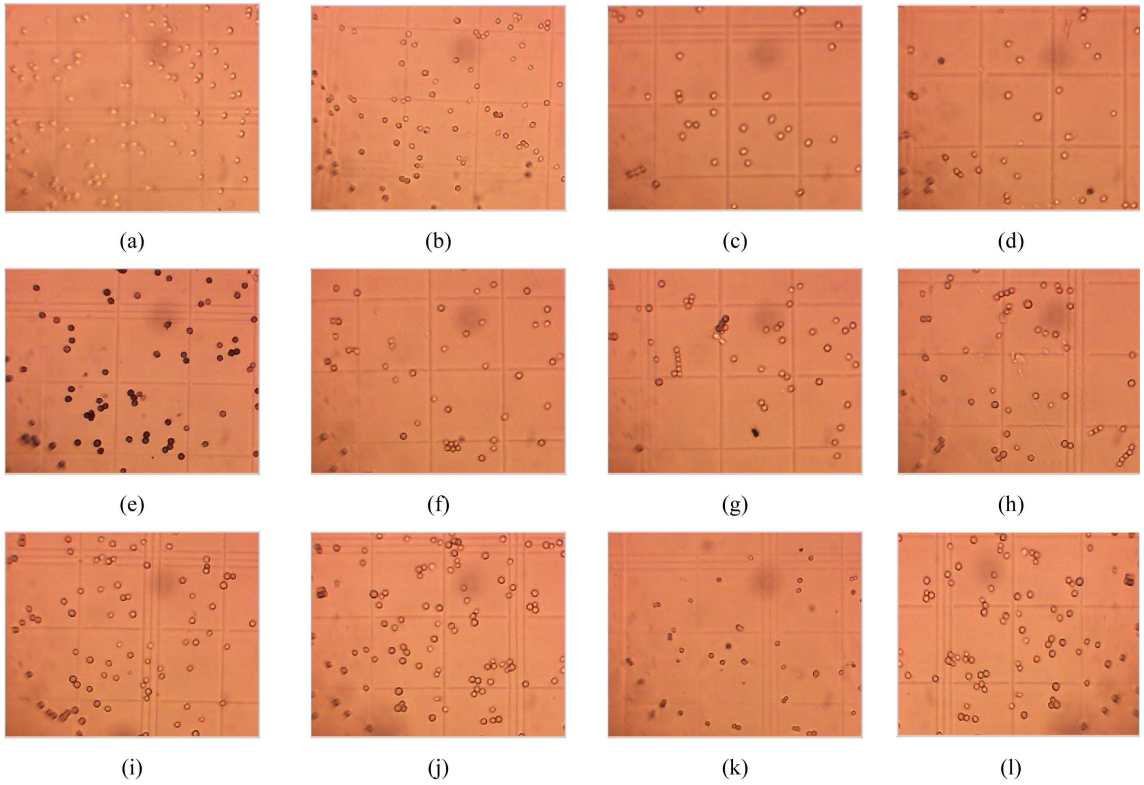

Figure 1. Fungal spores counting using a Hemocytometer slide under light microscope using a digital microscope eye piece with a magnification 400×: (a) *Geotrichum candidum*; (b) *Aspergillus ochraceus*; (c) *A. alliaceus*; (d) *A. oryzae*; (e) *A. niger*; (f) *A. nidulans*; (g) *Emericella nidulans*; (h) *A. flavus*; (i) *A. glaucus*; (j) *Penicillium* sp.; (k) *Rhizopus stolonifer* and (l) *A. flavipes*.

Table 1. Spores suspension counts of fungal strains.

Fungal strains	Spores count (×10^6/ml) (spore suspension)	Solution A (ml) (spore suspension)	Solution B (ml) (saline solution)
Geotrichum candidum	20.50	0.049	0.951
Aspergillus ochraceus	20.00	0.050	0.950
Aspergillus alliaceus	9.50	0.015	0.895
Aspergillus oryzae	19.00	0.053	0.947
Aspergillus niger	22.00	0.045	0.955
Aspergillus nidulans	60.00	0.017	0.983
Emericella nidulans	17.50	0.057	0.943
Aspergillus flavus	14.00	0.071	0.929
Aspergillus glaucus	9.00	0.012	0.888
Penicillium sp.	27.50	0.036	0.964
Mucor sp.	7.00	0.029	0.857
Rhizopus stolonifer	12.00	0.083	0.917
Aspergillus flavipes	26.00	0.038	0.962

The volume of solution A (spore suspension) was added to the volume of solution B (saline solution) for adjustment the fungal spores count to give a final 1×10^6 spores/ml using a micropipette (Micro Volume pipettor-Accumax A made in China).

All fungal strains did not give gas in the Durham's fermentation tube, but small air pebbles were observed under the fungal growth in case of *Geotrichum candidum*, *Aspergillus ochraceus*, *Emericella nidulans*, *Penicillium* sp., *Mucor* sp. and *Aspergillus flavipes* (**Figure 2**). It was also observed that, *A. nidulans* could produce a soluble red pigment which dissolved in the medium after 10 days of incubation. The finding which reported by Kure *et al*. [28] could explain the formation of gas where *G. candidum* is able to grow in environments with high levels of CO_2.

As Jarlsberg cheese releases higher amounts of carbon dioxide than Norvegia cheese, Jarlsberg cheese may provide an environment that selects for *G. candidum*. Similar results were obtained by Kure and Skaar [15] who reported that, *P. roqueforti* was capable of growth in an atmosphere with high levels of carbon dioxide. The growth was unaffected, or slightly stimulated, by high levels of CO_2, especially when levels of O_2 were low. This species is also resistant to weak acid preservatives. This may explain why *P. roqueforti* var. *roqueforti* is the dominant species with 39.5% of the total strains.

3.3. Efficiency of the Fungal Strains for Proteolysis

All fungal strains were tested for proteolysis on tow cultivation media. The first one was done on plates of modified PDA medium and incubated at 25˚C for 4 days. It was found that, fungal growth was very strong and caused a very rapid clear zone and was not suitable for enzymes activity evaluation. Thus the further experiments were controlled and examined every day on the second medium of modified Czapek agar (MCA). Also, all tested strains were inoculated on plates containing Czapek agar (CA) medium without skim milk addition as a control. Results of proteolysis were presented in **Table 2**. All tested fungal strains showed positive results. The fungal growth diameters were corresponding to clear zone diameter and it was varied between 16.40 to 94.53 mm in the cultures of *Aspergillus nidulans* and *Rhizopus stolonifer*, respectively. Also, *Geotrichum candidum*, *A. alliaceus*, *A. oryzae* and *E. nidulans* achieved the following values 21.76, 35.97, 26.71 and 31.87, respectively (**Figure 3**).

The presence of casein in the cultural medium caused a strong growth comparing with the growth on control. Changing of growth resulting from casein milk addition was in the highest values in the cultures of *Rhizopus stolonifer*, *A. flavus* and *A. niger* being 87.81, 58.32 and 41.58 mm, respectively. On the other hand, the lowest values were achieved by *A. nidulans* and *Geotrichum candidum* being 7.09 and 13.71 mm. Florez *et al*. [29] reported that, *Geotrichum candidum* is commonly found in mould-ripened cheese varieties. The strains consume lactate and produce several enzymes that break down proteins and fats. These processes involved in the softening of the cheese matrix and in the formation of aroma components (e.g., alcohols, methyl ketones, esters and various sulphides) in mature cheeses. *P. roqueforti* was also found in similar numbers of *G. candidum* suggesting that they also play a significant role in maturation of Cabrales cheeses.

Proteolysis is the most important event and the most complex for cheese texture by hydrolyzing the *para*-casein matrix which gives cheese its structure and by increasing the water binding capacity of the curd (*i.e*. to the new α-carboxylic and α-amino groups produced on cleavage of peptide bonds) [2] [5] [30]. However, the major role of proteolysis in cheese flavour is in the production of amino acids which act as precursors for a range of catabolic reactions which produce many important volatile flavour compounds. Small peptides produced from

Figure 2. Efficiency of the fungal strains for lactose assimilation (glycolysis).

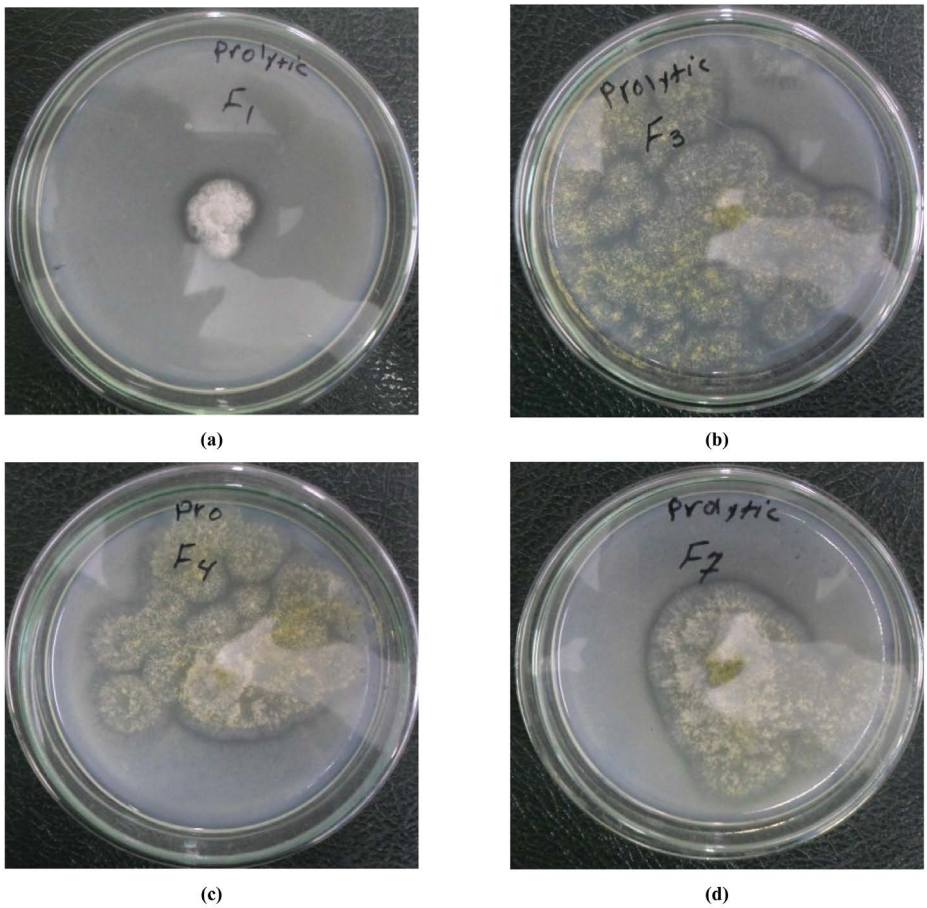

Figure 3. A positive result of casein proteolysis using (a) *Geotrichum candidum*; (b) *A. alliaceus*; (c) *A. oryzae* and (d) *E. nidulans* grown on a modified Czapek agar (MCA) medium.

Table 2. Efficiency of the fungal strains for proteolysis.

Fungal strains	Fungal growth diameter (mm) on CA medium as a control	Fungal growth diameter (mm) on MCA medium	Changing of growth* resulting from milk casein addition (mm)	Clear zone diameter (mm)
Geotrichum candidum	2.53	16.24	13.71	21.76
Aspergillus ochraceus	2.82	33.81	30.99	37.57
Aspergillus alliaceus	3.25	32.44	29.19	35.97
Aspergillus oryzae	3.75	22.86	19.11	26.71
Aspergillus niger	3.55	45.13	41.58	53.06
Aspergillus nidulans	3.58	10.67	7.09	16.40
Emericella nidulans	2.57	28.54	25.97	31.87
Aspergillus flavus	2.57	60.89	58.32	65.31
Aspergillus glaucus	1.24	33.20	31.96	39.79
Penicillium sp.	0.95	21.33	20.38	25.59
Mucor sp.	2.53	34.12	31.59	39.70
Rhizopus stolonifer	4.75	92.56	87.81	94.53
Aspergillus flavipes	1.02	30.90	29.88	35.41

*Changing of growth (mm) = growth diameter on MCA medium (mm) − growth diameter on CA medium as a control (mm).

hydrolysis of casein, can be broken down into short chain amino acids, peptides, amines, alcohols and sulphur containing compounds, which contribute to flavour formation, by proteinases and peptidases [3] [26] [27] [31] [32]. This observation is in agreement with the results of those [13] [14] [33] who reported that, during the aging of Kefalograviera, Cheddar, Domiati and Feta cheeses, the protein matrix was converted to a smoother structure and softening occurs. These changes are probably due to proteolysis of α_{s1}-casein, mainly by residual coagulant.

3.4. Efficiency of the Fungal Strains for Lipolysis

Lipids in foods may undergo hydrolytic or oxidative degradation. However, in cheese, oxidative changes are very limited due to the low oxidation/reduction potential (about −250 mV). However, triglycerides in all cheese varieties undergo hydrolysis by the action of lipases, which result in the liberation of fatty acids in cheese during ripening [25] [26].

After Ras cheese manufactured and completely salting process, the Ras cheese wheels were stored at about 15˚C for one month in a commercial ripening room, then the Ras cheese wheels were transferred in a digital incubator at 60˚F (15.5˚C) for another two months, thereafter samples were taken after one, two and three months. The storage of Ras cheese at this temperature accelerates lipolysis. Similar results were obtained by Mahony *et al*. [34] who studied the effect of temperature from 4˚C to 12˚C for ripened of commercial Cheddar cheeses for a total of 270 days. They found that, the levels of total and individual free fatty acids increased with increasing ripening temperature and progressive ripening time.

Increasing ripening temperature and time resulted in increases in the levels of short-(C4:0-C8:0), medium-(C10:0-C14:0) and long-(C16:0-C18:3) chain of free fatty acids. The results also suggested that the use of higher temperatures during the early stages of ripening (1 to 60 day) was most effective at accelerating lipolysis. Cheddar cheese ripened at low temperature (4˚C) did not attain the flavour and aroma characteristics typical of mature Cheddar cheese.

All the tested fungal strains were inoculated on plates containing Czapek agar (MCA) medium with milk fat addition as a control and incubated at 25˚C. Also, all tested species were inoculated on plates containing Czapek agar (CA) medium without milk fat addition as a control. Data in **Table 3** and **Figure 4** showed that, all strains gave positive results for lipolysis except *Aspergillus nidulans*. The fungal growth diameter was varied between 9.17 to 30.67 mm in the case of *A. niger* and *A. alliaceus*, respectively. The presence of fat milk in the cultural medium caused a changing of growth in the case of *A. glaucus*; *A. alliaceus* and *A. flavus* being 28.11; 27.42 and 25.19 mm, respectively. On the other hand *A. niger*, *Rhizopus stolonifer* and *A. flavipes* achieved lowest values being 5.62, 10.80 and 11.82 mm, respectively.

Results showed also that fungus *A. nidulans* gave negative reaction, but the changing of growth resulting from milk fat addition was 17.70 mm when compared with the value of control (3.58 mm). It may be due to the nutrients of milk fat addition such as fat and protein. Similar results were obtained by Hayaloglu and Kirbag [9] who reported that, *G. candidum* affects the cheese biochemistry during ripening. It releases lipases and proteases into the cheese matrix.

Lipolysis is particularly extensive in hard Italian cheese and blue mould chesses and is essential to correct flavour development in these cheeses. Extensive lipolysis is considered undesirable in other types of cheese varieties such as Cheddar, Gouda and Swiss cheeses; high levels of fatty acids in these cheeses lead to rancidity. However, low concentrations of free fatty acids contribute to the flavour of these cheeses, particularly when they are correctly balanced with the products of proteolysis or other reactions [27]. However, the most lipolytic organisms associated with cheese are *Penicillium* spp., which grow on or in mould-ripened varieties. *Penicillium roqueforti*, which causes the extensive lipolysis in blue cheese, possesses two lipases with pH optima of 7.5 - 8 and 9 - 9.5, respectively. *Penicillium camemberti* produces an extracellular lipase that is optimally active on tributyrin at pH 9 and 35˚C [14] [25] [26].

Malek *et al*. [35] isolated 35 strains of *Enterococcus faecium* isolated from two Egyptian cheeses (“Ras” and “Domiati”) in order to determine their ability for acidification, proteolysis and lipolysis activities, autolysis, texture, flavour development, antibiotic resistance and β-haemolytic activity. They demonstrate that, some indigenous strains of *E. faecium* displayed interesting technological properties for cheese manufacture, together with good safety characteristics. They could be useful for the manufacture of typical products in Egypt and Middle East.

Table 3. Efficiency of the fungal strains for lipolysis.

Fungal species	Fungal growth diameter (mm) on CA medium as a control	Lipolysis on MCA medium		
		Test result	Growth diameter (mm)	Changing of growth* resulting from milk fat addition (mm)
Geotrichum candidum	2.53	+	16.95	14.42
Aspergillus ochraceus	2.82	+	21.76	18.94
Aspergillus alliaceus	3.25	+	30.67	27.42
Aspergillus oryzae	3.75	+	18.81	15.00
Aspergillus niger	3.55	+	9.17	5.62
Aspergillus nidulans	3.58	-	21.28	17.70
Emericella nidulans	2.57	+	30.23	17.66
Aspergillus flavus	2.57	+	27.76	25.19
Aspergillus glaucus	1.24	+	29.35	28.11
Penicillium sp.	0.95	+	23.95	23.00
Mucor sp.	2.53	+	19.20	16.76
Rhizopus stolonifer	4.75	+	15.55	10.80
Aspergillus flavipes	1.02	+	12.84	11.82

*Changing of growth (mm) = growth diameter on MCA medium (mm) − growth diameter on CA medium as a control (mm).

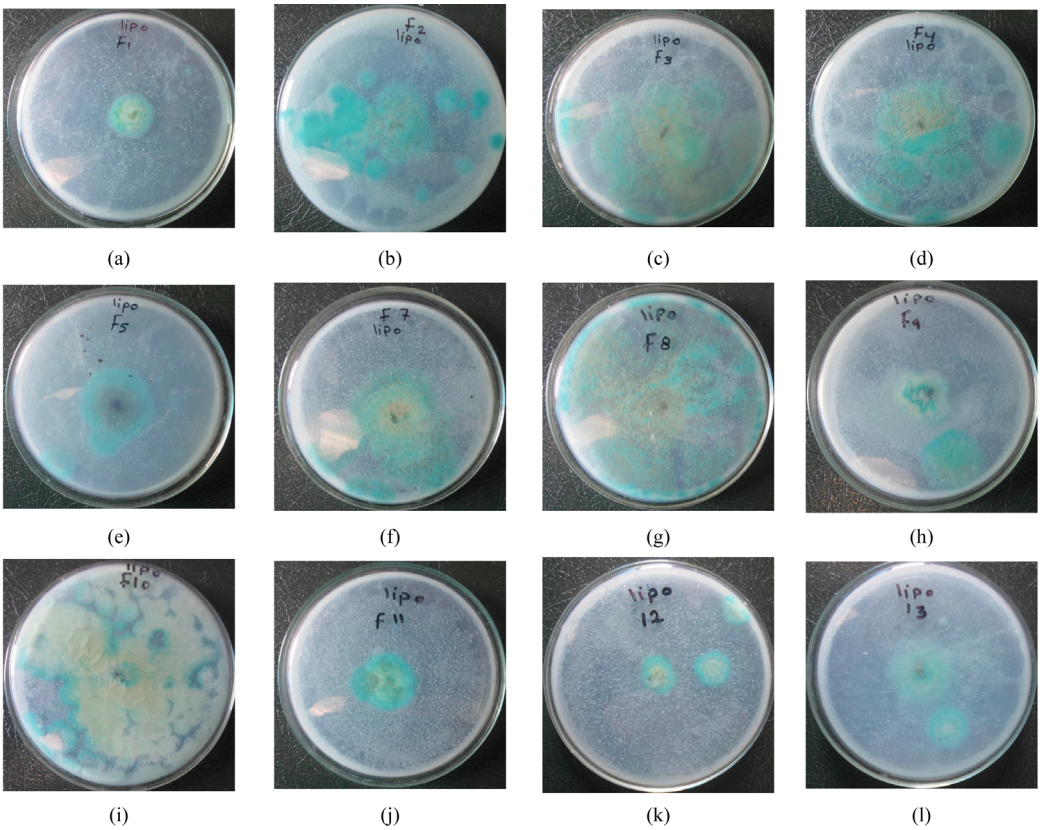

Figure 4. A positive result of lipolysis using fungal species grown on a modified Czapek agar (MCA) medium; (a) *Geotrichum candidum*; (b) *Aspergillus ochraceus*; (c) *A. alliaceus*; (d) *A. oryzae*; (e) *A. niger*; (f) *Emericella nidulans*; (g) *A. flavus*; (h) *A. glaucus*; (i) *Penicillium* sp.; (j) *Mucor* sp.; (k) *Rhizopus stolonifer* and (l) *A. flavipes*.

3.5. Chemical Composition of Ras Cheese during Storage

The moisture percentage (M%) was 38.86% in fresh Ras cheese wheels (**Table 4**), this value decreased to 34.67% after salting (after 45 day). This value gradually decreased during storage period (3 months at 15.5 C) from 31.29% in the first month to 30.25% in the third month. Dry matter percentage (DM%) was corresponded to moisture and took a reserve pattern of moisture. The total solids gradually increased towards the end of storage period. These findings are in agreement with those Kheadr *et al*. [2]; Tejada *et al*. [36]; Abdalla *et al*. [37]; Hamid and Abdelrahman [38]; Habib [14] and El-Fadaly, *et al*. [39] who reported that, the total solids contents of cheese increased during storage period. This increase was due to continuous loss of moisture from the crud a result of lactic acid development which caused crud contraction. However, the decrease in dry matter in the third month was possibly due to proteolytic effect of microorganisms on the protein and composed volatile components such as ammonia and volatile fatty acids into the ripening rooms.

Fat percentage was 24.05% in fresh Ras cheese wheels, this value increased to 25.89% after salting (after 45 day), this increase was due the lost of water and the increasing of dry matter. During ripening period the values of fat in Ras cheese wheels were 30.53%, 35.98% and 32.38% after the 1st, 2nd and 3rd month. The increasing of fat percentage was due to the fungal enzymes activities specially lipase (**Table 2**). These results are in accordance with the findings of Kheadr *et al*. [2]; Habib [14] and El-Fadaly, *et al*. [39] and in disagreement with those Hamid and Abdelrahman [38] and Abdalla *et al*. [37] who reported the decrease in fat content during storage period.

During cheese ripening the lost of weight and the decrease in dry matter was occurred. This decrease was due to the complete oxidation of lactate to CO_2 and H_2O and deamination of amino acids to the corresponding ketoacid and NH_3 [14]. In addition, volatile compounds which composed during ripening period also caused the decrease in dry matter. This compounds such as; hydrogen sulphide and methanethiol (resulting from decomposition of sulphur amino acids); ketones; methyl ketones; aldehydes; primary alcohols (e.g. ethanol); other aliphatic primary alcohols (e.g. 1-butanol, 1-pentanol and 1-hexanol); branched-chain primary alcohols (e.g. 2-methyl-1-butanol, 2-methyl-1-propanol 3-methyl-1-butanol and 3-Methyl-1-butanol) secondary alcohols (e.g. 2-propanol and 2-butanol); butanone, diacetyl, phenol (a major flavour compound in surface-ripened cheeses) and free fatty acids containing four carbons or more can originate from the lipolysis of milk fat or from breakdown of amino acids [1].

The highest value of F/DM% was 46.42% after 3 months of storage. The highest values of total nitrogen (TN%), soluble nitrogen (SN%) and non-protein nitrogen (NPN%) percentages of Ras cheese wheels were 4.18%; 43.97% and 18.98%, respectively. These values tended to increase with advanced storage. This increase was due to the fungal enzymes activities specially casinease and protease (**Table 3**). Awad *et al*. [40] reported the chemical composition of Ras cheese and they found that, moisture was % 47.46, fat was % 36.00 and protein was % 26.78. Dabiza and El-Deib [11] reported the chemical analysis of traditional Ras cheese and they found that, total nitrogen, soluble nitrogen and SN/TN were 16.01, 7.31 and 7.4, respectively.

Similar trends were obtained by El-Soda *et al*. [41]; Hassan *et al*. [42]; Mohedano *et al*. [43]; Osman and Abbas [44]; Abou-Donia [23]; Awad *et al*. [45]; Osman [46]; Habib [14] and El-Fadaly, *et al*. [13]. The proteolysis indices expressed as SN/TN and NPN/TN. The values of both were gradually increased during ripening period. The role of lipolysis and proteolysis in improving quality of Ras cheese was demonstrated by Hattem *et*

Table 4. Chemical composition of Ras cheese during storage.

Storage periods	Chemical examination of Ras cheese wheels						
	M%	DM%	F%	F/DM%	TN%	SN%	NPN%
Fresh	38.86	61.14	24.05	39.33	3.15	9.99	4.98
After salting	34.76	65.24	25.89	39.69	3.22	12.01	7.96
After 1 month	31.29	68.71	30.53	44.43	3.33	16.98	9.99
After 2 month	30.59	69.41	35.98	45.31	3.50	30.00	14.98
After 3 month	30.25	69.75	32.38	46.42	4.18	43.97	18.98

Where: M% = Moisture percentage; DM% = Dry Matter percentage; F% = Fat percentage; F/DM% = Fat on Dry Matter percentage; TN% = Total Nitrogen percentage; SN% = Soluble Nitrogen percentage and NPN% = Non-Protein Nitrogen percentage.

al. [12]. This role could be considered from the values of TVFA, SN/TN and NPN/TN which were greatly correlated with the sensorial properties of the mature cheese.

4. Conclusion

It could be concluded that, all 13 fungal strains 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. All tested fungal strains showed positive results for casein hydrolysis and the presence of casein in the cultural medium caused a strong growth. Also, all strains gave positive results for lipolysis except *Aspergillus nidulans*. 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 to reach 35.98 after two months. Also, total nitrogen, soluble nitrogen and non-protein nitrogen percentages reached to the highest values with advanced storage.

References

[1] Engels, W.J.M., Dekker, R., Jong, C., Neeter, R. and Visser, S. (1997) A Comparative Study of Volatile Compounds in the Water-Soluble Fraction of Various Types of Ripened Cheese. *International Dairy Journal*, **7**, 255-263. http://dx.doi.org/10.1016/S0958-6946(97)00003-4

[2] Kheadr, E.E., Vuillemard, J.C. and El-Deeb, S.A. (2003) Impact of Liposome-Encapsulated Enzyme Cocktails on Cheddar Cheese Ripening. *Food Research International*, **36**, 241-252. http://dx.doi.org/10.1016/S0963-9969(02)00166-7

[3] Amos, L.M. (2007) Enzymes from Yeast Adjuncts in Proteolysis during Cheddar Cheese Ripening. M.Sc. Thesis, The University of the Free State, Bloemfontein.

[4] Beresford, T.P., Fitzsimons, N.A., Brennan, N.L. and Cogan, T.M. (2001) Recent Advances in Cheese Microbiology. *International Dairy Journal*, **11**, 259-274. http://dx.doi.org/10.1016/S0958-6946(01)00056-5

[5] Cogan, T.M. and Beresford, T.P. (2002) Microbiology of Hard Cheese. In: *Dairy Microbiology Handbook*, 3rd Edition, John Wiley and Sons, Inc., New York. http://dx.doi.org/10.1002/0471723959.ch11

[6] McSweeney, P.L.H. (2004) Biochemistry of Cheese Ripening: Symposium Contribution. *International Journal of Dairy Technology*, **57**, 127-144. http://dx.doi.org/10.1111/j.1471-0307.2004.00147.x

[7] Gripon, J.C. (1993) Mould Ripened Cheeses. In: Fox, P.F., Ed., *Cheese*: *Chemistry*, *Physics and Microbiology*, Vol. 2, Chapman & Hall, London.

[8] Florez, A.B. and Mayo, B. (2006) Microbial Diversity and Succession during the Manufacture and Ripening of Traditional, Spanish, Blue-Veined Cabrales Cheese, as Determined by PCR-DGGE. *International Journal of Food Microbiology*, **110**, 165-171. http://dx.doi.org/10.1007/978-1-4615-2648-3_4

[9] Hayaloglu, A.A. and Kirbag, S. (2007) Microbial Quality and Presence of Moulds in Kuflu Cheese. *International Journal of Food Microbiology*, **115**, 376-380. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.04.016

[10] El-Soda, M. and Pandian, S. (1991) Recent Developments in Accelerated Cheese Ripening. *Journal of Dairy Science*, **74**, 2317-2335. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.12.002

[11] Dabiza, N. and El-Deib, K. (2007) Biochemical Evaluation and Microbial Quality of Ras Cheese Supplemented with Probiotic Strains. *Polish Journal of Food and Nutrition Sciences*, **57**, 255-300. http://dx.doi.org/10.3168/jds.S0022-0302(91)78405-1

[12] Hattem, H.E., Taleb, A.T., Manal, A.N. and Hanaa, S.S. (2012) Effect of Pasteurization and Season on Milk Composition and Ripening of Ras Cheese. *Journal of Brewing and Distilling*, **3**, 15-22. http://dx.doi.org/10.5897/JBD11.010

[13] El-Fadaly, H., El-Kadi, S.M., Hamad, M.N.F. and Habib, A. (2015) Isolation and Identification of Egyptian Ras Cheese (Romy) Contaminating Fungi during Ripening Period. *Journal of Microbiology Research*, **5**, 1-10.

[14] Habib, A.A.A. (2014) Studies on Fungal Decontamination of Ras Cheese during Storage by Using Some Natural Substances. Master's Thesis, Microbiology Department, Faculty of Agriculture, Damietta University, Damietta.

[15] Kure, C.F. and Skaar, I. (2000) Mould Growth on the Norwegian Semi-Hard Cheeses Norvegia and Jarlsberg. *International Journal of Food Microbiology*, **62**, 133-137. http://dx.doi.org/10.1016/S0168-1605(00)00384-6

[16] Bridson, E.Y. (2006) The OXOID Manual. 9th Edition, Oxoid Limited, Basingstoke.

[17] Osman, M.M. (2004) Factors Affecting the Antifungal Properties of *Brevibacterium linens*. *International Dairy Journal*, **14**, 713-722. http://dx.doi.org/10.1016/j.idairyj.2003.12.010

[18] Pintado, J., Gonzalez, M.P. and Murado, M.A. (1997) Interactions between Pretreatment and Nutrient Concentrations

of Mussel Processing for Citric Acid Production. *Enzyme and Microbial Technology*, **20**, 544-549. http://dx.doi.org/10.1016/S0141-0229(96)00199-8

[19] APHA, American Public Health Association (1998) Standard Methods for the Examination of Water and Wastewater. 20th Edition, APHA, Inc., New York.

[20] Skerman, V.B.D. (1967) A Guide to the Identification of the Genera of Bacteria. 2nd Edition, The Williams & Wilkins Co., Baltimore.

[21] Ronald, M.A. (2006) Hand Book of Microbilogical Media for the Examination of Food. CRC Taylor and Francis Group, Boca Raton, London and New York.

[22] Youssef, E.H. (1966) Studies on Ras Cheese. Master's Thesis, Ain Shams University, Cairo.

[23] Abou-Donia, S.A. (2002) Recent Development Ras Cheese Research. *Egyptian Journal of Dairy Science*, **30**, 155-166.

[24] Ling, E.R. (1963) Dairy Chemistry: Volume 2. 3rd Edition, Charman and Hall Ltd., London.

[25] McSweeney, P.L.H. and Sousa, M.J. (2000) Biochemical Pathways for the Production of Flavour Compounds in Cheeses during Ripening: A Review. *Le Lait*, **80**, 293-324. http://dx.doi.org/10.1051/lait:2000127

[26] McSweeney, P.L.H. (2004) Biochemistry of Cheese Ripening. *International Journal of Dairy Technology*, **57**, 124-144. http://dx.doi.org/10.1111/j.1471-0307.2004.00147.x

[27] Hassan, F.A.M., Abd El-Gawad, M.A.M. and Enab, A.K. (2013) Flavour Compounds in Cheese. *Research on Precision Instrument and Machinery*, **2**, 15-29.

[28] Kure, C.F., Wasteson, Y., Brendehaug, J. and Skaar, I. (2001) Mould Contaminants on Jarlsberg and Norvegia Cheese Blocks from Four Factories. *International Journal of Food Microbiology*, **70**, 21-27. http://dx.doi.org/10.1016/S0168-1605(01)00520-7

[29] Florez, A.B., Martin, P.A., Diaz, T.M.L. and Mayo, B. (2007) Morphotypic and Molecular Identification of Filamentous Fungi from Spanish Blue-Veined Cabrales Cheese and Typing of *Penicillium roqueforti* and *Geotrichum candidum* Isolates. *International Dairy Journal*, **17**, 350-357. http://dx.doi.org/10.1016/j.idairyj.2006.04.002

[30] Sousa, M.J., Ardo, Y. and McSweeney, P.L.H. (2001) Advances in the Study of Proteolysis during Cheese Ripening. *International Dairy Journal*, **11**, 327-345.

[31] Smit, G., Smit, B.A. and Engels, W.J.M. (2005) Flavour Formation by Lactic Acid Bacteria and Biochemical Flavour Profiling of Cheese Products. *FEMS Microbiology Reviews*, **29**, 591-610. http://dx.doi.org/10.1016/j.fmrre.2005.04.002

[32] Wit, M., Osthoff, G., Viljoen, B.C. and Hugo, A. (2005) A Comparative Study of Proteolysis in Cheddar Cheese and Yeast-Inoculated Cheddar Cheese during Ripening. *Enzyme and Microbial Technology*, **37**, 606-616. http://dx.doi.org/10.1016/j.enzmictec.2005.03.028

[33] Katsiari, M.C., Voutsinas, L.P., Alichanidis, E. and Roussis, I.G. (1998) Manufacture of Kefalograviera Cheese with Less Sodium by Partial Replacement of NaCl with KCl. *Food Chemistry*, **61**, 63-70. http://dx.doi.org/10.1016/S0308-8146(97)00113-1

[34] Mahony, J.A.O., Sheehan, E.M., Delahunty, C.M. and McSweeney, P.L.H. (2006) Lipolysis and Sensory Characteristics of Cheddar Cheeses Ripened Using Different Temperature-Time Treatments. *Le Lait*, **86**, 59-72. http://dx.doi.org/10.1051/lait:2005041

[35] Malek, R., El-Attar, A., Mohamed, M., Anwar, S., El-Soda, M. and Beal, C. (2012) Technological and Safety Properties Display Biodiversity among Enterococci Isolated from Two Egyptian Cheeses, "Ras" and "Domiati". *International Journal of Food Microbiology*, **153**, 314-322. http://dx.doi.org/10.1016/j.ijfoodmicro.2011.11.019

[36] Tejada, L., Abellan, A., Cayuela, J.M., Cacha, A.M. and Salguero, J.F. (2008) Proteolysis in Goats' Milk Cheese Made with Calf Rennet and Plant Coagulant. *International Dairy Journal*, **18**, 139-146. http://dx.doi.org/10.1016/j.idairyj.2007.08.010

[37] Abdalla, M.O.M., Kheir, S.E.O. and Elowni, O.A.O. (2011) Effect of Storage Period on the Quality of Sudanese White Cheese (Gibna Bayda) Manufactured with *Solanum dubium* Extract. *University of Khartoum Journal of Agricultural Sciences*, **19**, 202-217.

[38] Hamid, O.I.A. and Abdelrahman, N.A.M. (2012) Effect of Adding Cardamom, Cinnamon and Fenugreek to Goats Milk Crud on the Quality of White Cheese during Storage. *International Journal of Dairy Science*, **7**, 43-50. http://dx.doi.org/10.3923/ijds.2012.43.50

[39] El-Fadaly, H., Hamad, M.N.F., El-Kadi, S.M. and Habib, A. (2015) Effect of Clove Oil on Physicochemical and Microbiological Characteristics of Egyptian Ras Cheese (Romy) during Storage. *International Journal of Food Science and Nutrition Engineering*, **5**, 15-23.

[40] Awad, R.A., Salama, W.M. and Farahat, A.M. (2014) Effect of Lupine as Cheese Base Substitution on Technological and Nutritional Properties of Processed Cheese Analogue. *Acta Scientiarum Polonorum Technologia Alimentaria*, **13**,

55-64. http://dx.doi.org/10.17306/J.AFS.2014.1.5

[41] El-Soda, M., Ezzat, J., El-Abassy, F. and Wahba, A. (1990) Acceleration of Ras Cheese Ripening, II, Combination of Gross Proteolytic Agent with the Cell Free Extract of Some Lactobacilli. *Egyptian Journal of Dairy Science*, **18**, 183-193.

[42] Hassan, A.A., Mahran, G., Ismail, A.A. and El-Hofi, M.A. (1996) Studies on Ras Cheese Ripening with Enzymes Addition, 1-Preparation of Lipase and Protease from Bacteria and Its Addition to Ras Cheese Slurry. *Egyptian Journal of Food Science*, **24**, 373-399.

[43] Mohedano, A.F., Fernandez, J., Garde, S., Medina, M., Gaya, P. and Nunez, M. (1998) The Effect of the Cysteine Proteinase from *Micrococcus* sp. INIA 528 on the Ripening Process of Manchego Cheese. *Enzyme and Microbial Technology*, **22**, 391-396. http://dx.doi.org/10.1016/S0141-0229(97)00211-1

[44] Osman, A.A. and Abbas, F.M. (2001) Fate of *Lactobacillus acidophilus* La-5 and *Bifidobacterium lactis* Bb-12 in "Probiotic" Ras Cheese. *Proceedings of the* 8*th Egyptian Conference for Dairy Science and Technology*, Cairo, 3-5 November 2001, 653-665.

[45] Awad, S., El-Attar, A., Ayad, E.H.E. and El-Soda, M. (2003) Characteristic of Egyptian Market Ras Cheese; Sensory Evaluation, Rheological, Physico-Chemical Properties and Microbiological Analysis. *Egyptian Journal of Dairy Science*, **31**, 289-303.

[46] Osman, M.M. (2003) Acceleration of the Ripening and Flavour Development of Ras Cheese Using *Brevibacterium linens*. *Egyptian Journal of Dairy Science*, **31**, 159-172.