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Optimization of Polyunsaturated Fatty Acids (PUFAs) Production from Olive Cake by *Pseudomonas fluorescens* NBRC14160 using Response Surface Methodology and their Application in Kareish Cheese Manufacture

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Abstract

The aim of this study was to optimize the production of polyunsaturated fatty acids (PUFAs), using the response surface methodology (RSM), to use the produced PUFAs in kareish cheese processing. Plackett Burman design (PBD) was employed to screen media components that affect PUFAs development (glucose, olive cake, yeast extract, tryptone, $MgSO_4$, KH_2PO_4, NH_4Cl, agitation speed, incubation time, and pH), and results showed that olive cake and yeast extract, with confidence level > 98%, had a positive effect on PUFAs production. The central composite design (CCD) of the response surface methodology was used to optimize the selected parameters levels where maximum PUFAs production (1790 mg/l) was observed near the mid-point (0) values (concentrations) of olive cake (15 g/l), tryptone (7.5 g/L) and KH_2PO_4 (1.25 g/l). Polyunsaturated fatty acids account for 47.83 % of the total fatty acid profile, according to gas chromatography analysis of the collected PUFAs. The produced PUFAs was encapsulated using whey protein concentrate and maltodextrin, freeze dried, grinded and incorporated in Kareish cheese manufacture. The average particle size of a 0.005% suspension of oil microcapsules was 671.4 nm with a poly dispersity index of 0.611 indicating a moderate stability of the emulsion. The negative zeta potential of the microcapsules particles was -37.6 mv, which is identical to the -42 mv value recorded in the literature for oil emulsions stabilized by whey protein, maltodextrin, and K-carrageenan. The addition of 0.5% PUFAs-containing microcapsules to Kareish cheese increased antioxidative activity to 38.13 % compared to 30.14 % for the control, as well as Texture profile analysis (TPA) parameters including hardness, cohesiveness, gumminess, and chewiness. The elasticity of the Kareish cheese sample increased slightly by the addition of 0.5% microcapsules, but higher concentration tended to change the elasticity to a brittleness of the cheese structure.

Keywords: PUFAs, Production, RSM, *Pseudomonas fluorescens*, GC analysis, Antioxidant activity, UF, Kareish cheese.

1 Introduction

Vegetable oils are harmful to the environment, like petroleum oils they produce similar environmental effects. Hence, it is a known fact that the olive oil causes the production of large quantity of olive mill waste

(OMWs) as by-products to the environment. These by-products are harmful to the environment, both terrestrial and aquatic. In North Africa and Mediterranean regions as great producers of olive oil, however, many other countries are now producers, others used to be high producers of oil and these countries had faced and present producers will continue to face the environmental problems generated by oil mill wastes (Popoola et al 2017). Lipids rich in polyunsaturated fatty acids (PUFAs) are usually found in the oil of marine fatty fish such as mackerel, sardine, albacore tuna, and salmon.

Polyunsaturated fatty acids (PUFAs) are classified into two groups, based on the first double bond in the fatty acid chain, affined to the methyl terminus, these two groups are: omega-3 (ω-3) fatty acids, having its last double bond at the 3rd carbon atom and omega-6 (ω-6) fatty acids, having their last double bond at the 6th carbon atom and both of them cannot be synthesized spontaneously in the human body (Ander et al 2003). PUFAs, such as Omega-3 (ω-3) fatty acids, are important in human health, particularly for the brain, eye, and cardiovascular systems (Carballeira 2008). Furthermore, a diet with a healthy ω -3/ ω -6 ratio are suggested to reduce the risk of Alzheimer's disease, high cholesterol, and cancer (Sahin et al 2018, Simopoulos 2003). However, in the absence of docosahexaenoic acid (DHA), one of the most important omega-3 fatty acids in the human diet, irregular brain functions were observed. As a result of high consumption rate, there was a significant rise in demand all over the world. The enormous gap between PUFAs production and consumption has prompted many researchers to look for alternative sources of PUFAs production and optimization, especially for minor fatty acids, which have much more promising potential applications. One of the best new sources to fulfil this gap is the microbial sources, such as fungi, yeasts, algae and bacteria, due to its ability to accumulate large amounts of cellular lipids using the fatty acid synthase (FAS) system followed by a series of modification process like desaturases and elongases. *Pseudomonas fluorescence* strain is known to produces lipases, yielding extracellular fatty acids. Cellular lipids rich of naturally occurring PUFAs can reach up to 80% of their cell mass as a reserve storage material depending on different nutritional and physical factors that can affect the total production of lipids, which it can be very insightful for food industry and biotechnological applications, (Ochsenreither et al 2016). Because of microbial continuous supply for high pharmaceutical-grades of PUFAs as reported by Certik and Shimizu (1999), and Abd Elrazak et al (2013). For that, a comprehensive model that considers all of the main effective factors that affects the production rate on a single factor level and the interaction between these factors should be used for the optimization purposes. Many studies have been performed on the use of unsaturated fatty acids in the dairy industry. Beside their positive health impact on the human, they induced lipolysis and oxidative stability of the cheese product with minor changes in their technological, sensorial and functional qualities and organoleptic properties (During et al 2000, Gbassi et al 2012, Khalifa et al 2017). Thus, fresh and soft cheese could be considered as a vehicle for polyunsaturated fatty acids integration (Dal Bello et al 2017, Villamil et al 2021).

Therefore, the aim of this work was to optimize the production of PUFAs using *Pseudomonas fluorescens* NBRC14160, using response surface methodology, for the enhancement of kareish cheese by fortifying white cheese product by the produced PUFAs.

2 Materials and Methods

2.1 Microorganism

The strain of *Pseudomonas fluorescens* NBRC14160 (GenBank accession no. NR 113647.1) used in this study was previously isolated from Egyptian delta soil and identified using 16S rRNA gene sequencing (Yahia et al 2020). The culture was maintained on King's medium (APHA, 1992) slants at 4˚C and subcultured at monthly intervals. King's medium

has the following composition (g/l of distilled water): Proteose peptone 20, Dipotassium hydrogen phosphate, 1.5 Magnesium sulphate heptahydrate, 1.5 Agar 20 with final pH adjusted (at 25°C) to 7.2 ± 0.2.

For the preparation of standard inoculum, 50 ml of King's broth medium in 250 ml Erlenmeyer flasks were inoculated with a full loop of tested culture. Incubation was carried out using a rotary shaking incubator (Lab-line Ltd.) at 120 rpm for 24 h at 30˚C and used as the standard inoculum (7.0×10^5/ml viable cells) for shake flasks experiments.

2.2 Collection of ultrafiltered (UF) skim milk and cheese starters

Fresh skim UF-retentate was obtained from Animal Production Research Institute, Agriculture Research Center, Dokki, Egypt. Freeze dried cheese culture of *Lactococcus lactis subsp. lactis* and *Lactococcus lactis subsp. cremoris* (FD-VVS R 704) were obtained from Chr. Hansens's Lab, Denmark.

2.3 Effect of incubation time on PUFAs production by *Pseudomonas fluorescens* NBRC14160 on King's medium

For determination of the ability of *Pseudomonas fluorescens* NBRC14160 to produce PUFAs fatty acids as affected by incubation time, 50 ml of King's broth medium supplemented with 2% olive cake in 250 ml Erlenmeyer flasks were inoculated with a full loop of tested culture. Flasks were incubated in a rotary shaking incubator (Lab-line Ltd.) at the rate of 120 rpm for 72h at 30˚C. For detection of biomass and total PUFAs, 10 ml of the samples were taken at 2h intervals for 72 hours. Samples were centrifuged at 10000 rpm for 15 minutes under cooling at 4°C, and supernatant was collected to detect the total produced PUFAs (Frings et al 1972). All samples were carried out in triplicates.

2.4 Screening of factors affecting (PUFAs) production using Plackett-Burman Design (PBD)

The Plackett-Burman design was used to investigate the synergistic effects of nutritional and physical factors, such as carbon and nitrogen sources, mineral salts, agitation speed, incubation time, and pH, on production of PUFAs. Ten variables, at two levels, high and low, were chosen for this analysis, as shown in **Fig 1** and **Table 1.** The key variables chosen for this study were: glucose, olive cake, yeast extract, tryptone, MgSO4, KHPO4, NH4Cl, agitation speed, incubation time, and pH, based on the Plackett-Burman matrix design. **Table 2** illustrates the experimental design with the variable's name, and actual level. All media were inoculated with 5% of standard inoculum (7.0×10^5 cfu/ml) of *Pseudomonas fluorescens*. Inoculated flasks were incubated in shaker incubator (Lab line Ltd.) at 30˚C with shaking at different agitation speed rates ranged from 120 to 200 rpm according to the run number. The growth measurements and total PUFAs production assays were done after 48 h of incubation. "Design Expert® 12" Stat-Ease, Inc, Minneapolis, USA, was used to evaluate the experimental Plackett–Burman design.

2.5 Central Composite Design (CCD) of Response Surface Methodology (RSM) for optimization of media components

According to the results obtained from PBD, three factors were selected as the main significant factors (olive cake, yeast extract and KH_2PO_4). *Pseudomonas fluorescens* NBRC14160 was used in the central composite Design (CCD) of the response surface methodology (RSM) to statistically optimize 20 trials of process parameters. The experiment was designed, data was analyzed, and a quadratic model was built using the software "Design Expert" (Version 12, Stat-Ease Inc, Minneapolis, USA). **Tables 3 and 4** show the minimum and maximum ranges of variables investigated due to their values in actual form.

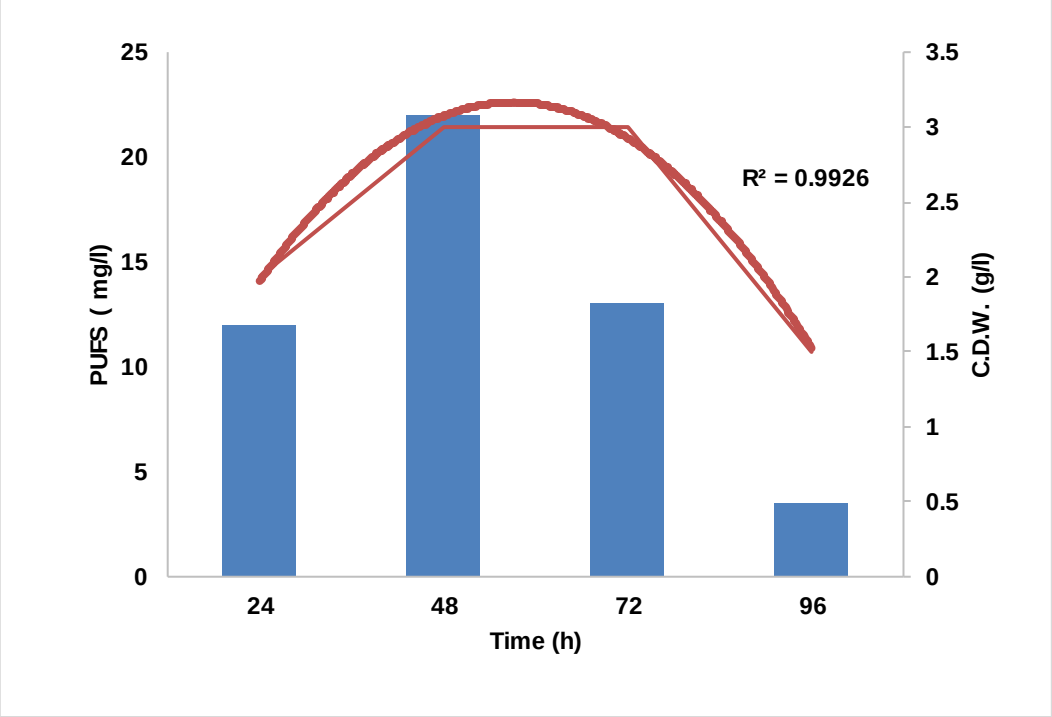


Fig 1. PUFAs production during 96 h of incubation at 30 °C on basal medium

Table 1. Levels of growth parameters tested in Plackett–Burman design

Variables	Symbols	Coded levels	
		-1 (Low)	+1 (High)
Glucose	(A)	10	30
Olive cake	(B)	10	20
Yeast extract	(C)	5	10
Tryptone	(D)	5	10
$MgSO_4$	(E)	0.5	2
KH2PO4	(F)	0.5	2
NH4Cl	(G)	0.5	2
Agitation	(H)	120	200
Incubation time	(J)	20	48
pH	(K)	6	8

Table 2. Plackett–Burman Design Matrix for growth parameters optimization experiment

Run order	Glucose (g/l)	Olive cake (g/l)	Yeast extract (g/l)	Tryptone (g/l)	$MgSO_4$ (g/l)	KH_2PO_4 (g/l)	NH_4CL (g/l)	Agitation (rpm)	Incubation Time °C	pH
1	20.0	15.0	7.5	7.5	1.25	1.25	1.25	160	22	7
2	10.0	10.0	5.0	10.0	0.5	2.0	2.0	120	24	8
3	30.0	20.0	5.0	10.0	2.0	2.0	0.5	120	20	8
4	10.0	10.0	5.0	5.0	0.5	0.5	0.5	120	20	6
5	10.0	20.0	5.0	10.0	2.0	0.5	2.0	200	24	6
6	20.0	15.0	7.5	7.5	1.25	1.25	1.25	160	22	7
7	10.0	10.0	10.0	5.0	2.0	2.0	0.5	200	24	8
8	20.0	15.0	7.5	7.5	1.25	1.25	1.25	160	22	7
9	30.0	20.0	5.0	5.0	0.5	2.0	0.5	200	24	6
10	10.0	20.0	10.0	10.0	0.5	0.5	0.5	200	20	8
11	10.0	20.0	10.0	5.0	2.0	2.0	2.0	120	20	6
12	20.0	15.0	7.5	7.5	1.25	1.25	1.25	160	22	7
13	30.0	10.0	10.0	10.0	2.0	0.5	0.5	120	24	6
14	30.0	10.0	5.0	5.0	2.0	0.5	2.0	200	20	8
15	30.0	10.0	10.0	10.0	0.5	2.0	2.0	200	20	6
16	30.0	20.0	10.0	5.0	0.5	0.5	2.0	120	24	8
17	20.0	15.0	7.5	7.5	1.25	1.25	1.25	160	22	7
18	20.0	15.0	7.5	7.5	1.25	1.25	1.25	160	22	7

The letter I is absent by default in the software.

Table 3. Levels of nutritional factors tested in CCD design

Variable	Symbol	Levels of the variables tested in CCD		
		(Low)	(Mid)	(High)
Olive cake	(A)	10	15	20
Yeast extract	(B)	5	7.5	10
KH_2PO_4	(C)	0.5	1.25	2

Table 4. CCD Design Matrix for media components optimization experiment

Run order	Coded levels		
	Olive cake (A) (g/l)	Yeast extract (B) (g/l)	KH_2PO_4 (g/l)
1	10.00	10.00	0.50
2	15.00	3.30	1.25
3	15.00	7.50	1.25
4	15.00	7.50	0.00
5	15.00	7.50	1.25
6	10.00	5.00	2.00
7	23.41	7.50	1.25
8	6.59	7.50	1.25
9	15.00	7.50	2.51
10	15.00	7.50	1.25
11	15.00	7.50	1.25
12	20.00	5.00	2.00
13	10.00	5.00	0.50
14	15.00	7.50	1.25
15	15.00	11.70	1.25
16	15.00	7.50	1.25
17	10.00	10.00	2.00
18	20.00	10.00	0.50
19	20.00	5.00	0.50
20	20.00	10.00	2.00

All media were inoculated with 5% of standard inoculum (1x 10^7 cfu/ml) of *Pseudomonas fluorescens*. After inoculation, flasks were placed in shaker incubator (Lab line Ltd.) at 30˚C with shaking at 160 rpm for 48h. The total PUFAs was used as the dependent variable or response after the experiments were completed (Y).

Multiple regression analysis was used to fit a second order polynomial equation to the results as follows:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$

Where, Y, is the predicted response, β_0 intercept, β_1, β_2, β_3, linear coefficients, β_{11}, β_{22}, β_{33}, squared coefficients, β_{12}, β_{13}, β_{23}, interaction coefficients. The level of fit of the second-order polynomial model equation was calculated using correlation coefficient ($R^{2)}$. To demonstrate the relationship between total PUFAs output and the experimental levels of each parameter used in this study, three-dimensional surface plots were generated to express the fitted polynomial equation.

2.6 Validation of growth parameters for lipids production optimization by *Pseudomonas fluorescens* NBRC14160

The validation of statistical model of growth parameters was done by carrying out the experiment at optimum values of the selected parameters (olive cake, yeast extract and KH_2PO_4) as determined from the model. Medium was prepared by adjusting pH to 7. After inoculation, flasks were incubated at 30˚C with shaking at 160 rpm. After 48 h, growth and total PUFAs were determined as described before. The obtained experimental values were compared to the model's expected values.

2.7 Extraction and determination of biomass and total PUFAs concentration

For the extraction of total PUFAs, the fermented medium was centrifuged at 10000 rpm for 15 min at 4˚C to obtain total PUFAs rich supernatant. According to Frings et al (1972), the supernatant was collected for total lipid determination. For biomass determination, cell pellets were collected, and biomass was determined. After extraction, produced PUFAs product was sterilized by autoclaving at 121 ºC / 20 min. then lyophilized for further studies.

2.8 Total PUFAs production parameters

Productivity (P) = concentration of PUFAs (mgl^{-1}) / fermentation time (h) = $mgl^{-1}h^{-1}$.
Total PUFAs yield coefficient relative to biomass ($Y_{p/x}$) (mgg^{-1}) = Amount of PUFAs produced (mgl^{-1}) / amount of biomass (gl^{-1})

2.9 Gas chromatography analysis for PUFAs

The fatty acids composition was determined by the conversion of oil to fatty acid methyl esters (FAMEs) according to the modified method described by Zahran and Tawfeuk (2019). FAMEs were separated with an HP 6890 plus gas chromatography (Hewlett Packard, USA), using a capillary column Supelco™ SP-2380 (60 m×0.25 mm×0.20 µm), (Sigma-Aldrich, USA), Detector (FID) and the injector and detector temperatures were 250°C. The column temperature was 140°C (hold for 5 min) and raised to 240°C, at rate of 4°C/min, and holds at 240°C for 10 min. The carrier gas was helium at flow rate 1.2 mL min^{-1}. FAMEs were identified by comparing their relative and absolute retention times to those authentic standards of FAMEs (SupelcoTM 37component FAME mix). The fatty acid composition was reported as a relative percentage of the total peak area.

2.10 Oil Microencapsulation

Preparation of oil emulsion

The oil emulsion was formulated as follows: 10 wt % oil, 10 wt % whey protein concentrate, 10 wt % maltodextrin and 70 % water. The hydrated solutions of wall materials (whey protein concentrate and maltodextrin)

once prepared, were being stirred at room temperature (25˚C) for 2 h to confirm a full hydration of the polymer particles (Heinzelmann et al 2000). Then oil was added and dispersed using a digital ULTRA-TURRAX® T18 homogenizer (IKA, Germany), at speed of 15 x 10^3 rpm/5 min.

Freeze drying of oil emulsion

The freeze-drying process was performed in a laboratory scale freeze dryer (Lyophilyzer, ZiRBUS technology, Vaco 5, Germany). The emulsion was pre-frozen at −22°C for 24 h before lyophilization at a pressure of 20 Pa for 48 h until reaching a final moisture content of 3%.

2.11 Measurement of particle size and zeta potential

The particle size and zeta potential of the droplets in the emulsions were determined using Zetasizer var. 704 instruments (Malvern Instruments, Malvern, UK) as described by Agustinisari et al (2020). For measuring size distribution and zeta potential, the sample was sonicated using sonicator equipment (Q 500, Power Rating: 500 watts) for 15 min. just before assessment. Sample was diluted with ultrapure water before measuring its light scattering for a laser beam (633 nm) at an angle of 173 ° at 25˚C over time intervals. The changes in laser beam scattering versus time was used to determine the particle size distribution. The mean particle diameter (the scattering intensity-weighted mean diameter, Z-average) and polydispersity index (PDI) were calculated from the particle size distribution. The PDI value provides a measure of the narrowness of the particle size distribution, with values ≤0.1 indicating a very narrow distribution.

2.12 Manufacturing of Kareish cheese

Kareish cheese was made using the method described by Maubois et al (1987). UF-retentate was heated to 72°C for 3-5 min and then cooled to 42°C. Freeze dried microencapsulated oil emulsion (microcapsules) was mixed with UF-retentate at concentrations of zero % (control, T0), 0.5% (T1), 1% (T2) and 1.5% (T3) using the electric blender (Molinex blender). The four treatments were salted with a concentration of 1.5 % and inoculated with 2% cheese culture and packaged in 75 ml plastic cups. All cups were incubated at 42°C till coagulation. The resultant cheese was analyzed when fresh, and after 7,14 and 28 days of storage at (5± 2 °C).

2.13 Antioxidative activity of Kareish cheese

Free radical Scavenging activity (RSA) of the samples was measured using the method of Brand-Williams et al (1995) and expressed as percentage inhibition of the DPPH radical and was calculated by the following equation:

$$\text{RSA\%} = \frac{Abs\ control - Abs\ sample}{Abs\ control}\ X\ 100$$

2.14 Texture profile analysis of Kareish cheese

Cheese samples were subjected to texture profile analysis (TPA) using a Texture Analyzer (TMS-Pro, USA). The samples were subjected to two successive compressions (bites) at 25% deformation using a cylindrical probe of 20 mm diameter and 35 mm length at three different locations for each cheese sample. The speed of the crosshead was kept at 1mm/sec with a load cell of 25 N. Fracturability, Hardness 1 and Hardness 2, work carried out on the sample during the first bite (A1) and on the second bite (A2), cohesiveness (A2/A1), springiness (elasticity) and chewiness were calculated from the obtained TPA profiles as described by Lobato-Calleros et al (1997).

2.15 Sensory evaluation

Cheese samples were sensory scored by 10 panelists according to their consistency in attending as mentioned by ADSA (1987) for flavor (50 points), body and texture (35 points) and appearance (15 points).

2.16 Statistical analysis

The above-mentioned experiments' data were analyzed using one-way ANOVA with "Design Expert" software (Version 12, Stat-Ease Inc, Minneapolis, USA) at a significance level of 0.05 in shake flask experiments.

3 Results and Discussion

3.1 Effect of incubation time on PUFAs production by *Pseudomonas fluorescens* NBRC14160 on King's medium

Fig 1 represents the biological activity of total PUFAs production. The highest total PUFAs reached 22 mg/l after 48h. of incubation which is in agreement with the total biomass 3 g/l with correlation coefficient R^2 of 0.9926 which indicated that production of total PUFAs by *Ps. fluorescens* is growth dependent by 99.26%.

3.2 Statistical screening of nutritional factors using Plackett-Burman Design (PBD)

PBD design was conducted in 18 test runs at high and low levels for each variable to detect key factors affecting the production of PUFAs. With an F-value of 4.28, the one-way ANOVA revealed the model's significance. The main effects plot is shown in **Fig 2**, which is used in combination with the ANOVA analysis to determine the mean-level differences for all variables. The produced mean PUFAs for each factor level was connected by a line. Factors with horizontal line parallel to the X axis do indicate the main effects. However, factors with non-horizontal line are considered as the main affecting factors. Hence, olive cake (B), yeast extract (C), and KH_2PO_4 (F) are significant main effects for the PUFAs production.

KH_2PO_4 was found to have a positive impact on PUFAs production in this study as a source of phosphorus and potassium and as a coenzyme for PUFAs production. as recorded by Ghori et al (2011).

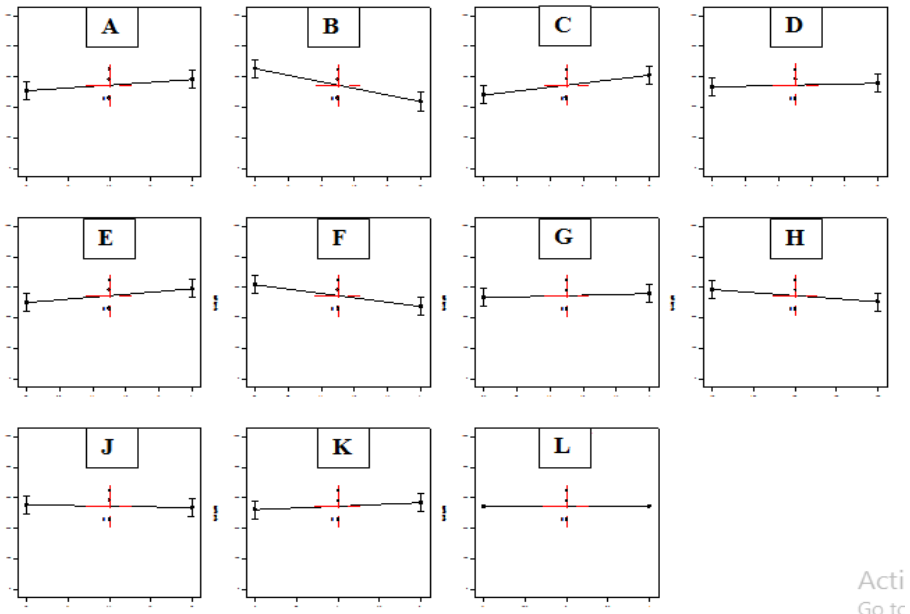


Fig 2. Main effects plot of PBD indicating that olive cake (B), yeast extract (C) and KH_2PO_4 (F) are the main significant media components affecting PHB production by *Pseudomonas fluorescens* NBRC 11640

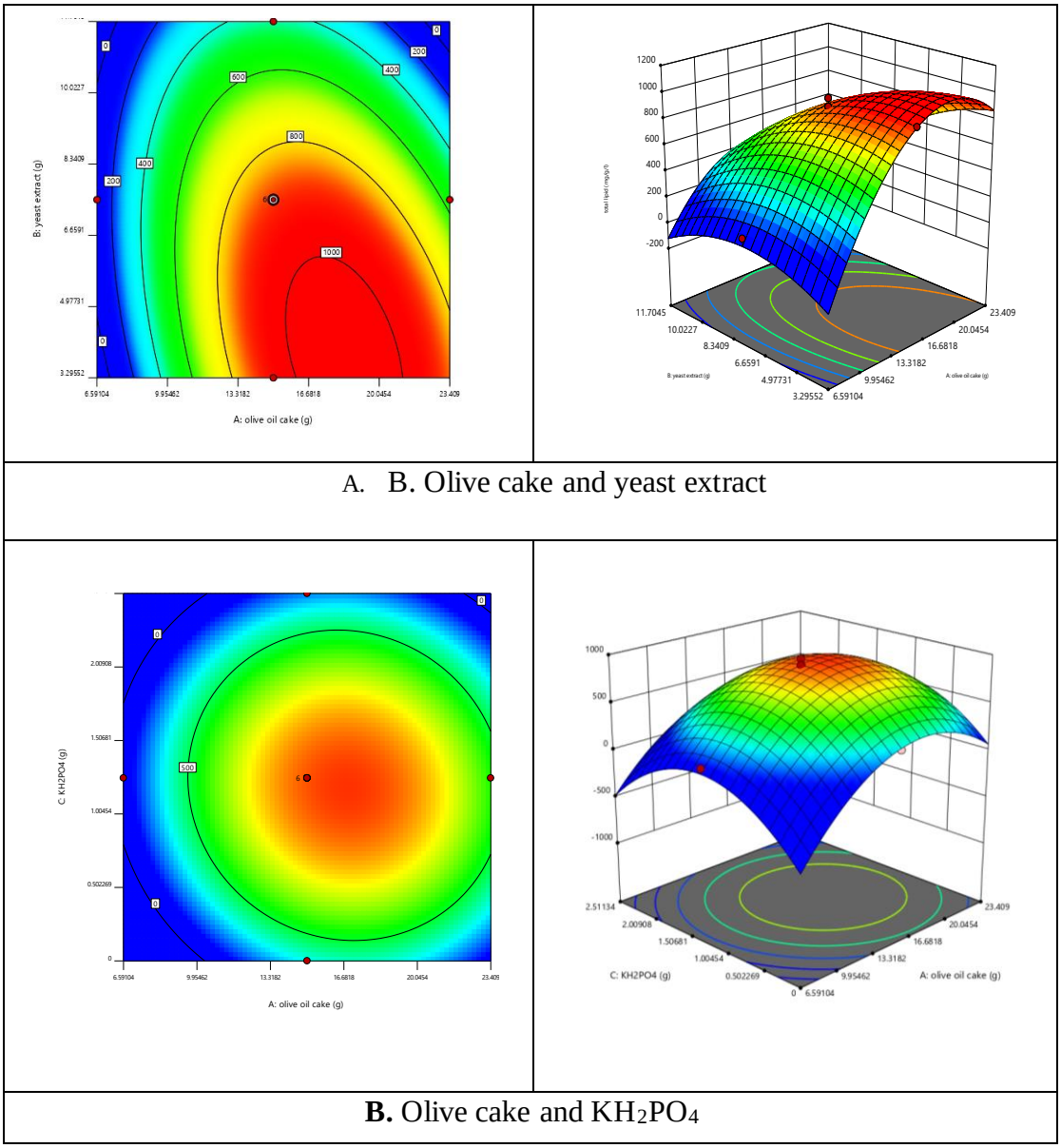
3.3 CCD Optimization of growth parameters affecting PUFAs production.

In this study, 20 experiments with different combinations of olive cake (A), yeast extract (B) and KH_2PO_4 (C) concentrations were performed at three different levels coded as −1, 0, and +1 are presented along with the actual and predicted responses**.** Additionally, the software suggested higher and lower factor levels than the coded levels to decrease the noise ratio. **Table 5** shows that the maximum production (1789.46mg/l) was achieved at run 5 in the presence of 15 g/L of olive cake, 7.5 g/L of yeast extract, 1.25 g/L of KH_2PO_4**. Fig 3** illustrates the relationship (surface response) between olive cake and other media components. **Fig 3A-B** describes the relationship between olive cake and yeast extract, olive cake and KH_2PO_4 on PUFAs production that led to a sharp increase in PUFAs production by *Pseudomonas* cells to reach 1789.46 mg/l (81.3 times) as compared to the basal one. F-test and ANOVA were conducted. The F-value for the model was 2.53 implied that the model was significant. The coefficient of determination R^2 of the model was 0.9765 and indicated that 97.65% of the total variations were explained by the model and revealed a good agreement between the experimental results and the predicted values calculated from the model. The final equation in terms of actual factors is as follows:

Y (total PUFAs) = -3097.91637 + 331.21099 olive cake + 233.09 yeast extract + 1069.05 KH_2PO_4 -7.19 olive cake * yeast extract - 5.76 olive cake * KH_2PO_4 - 9.80 yeast extract* KH_2PO_4 -8.06 olive cake^2-11.59 yeast extract^2 -374.34 KH_2PO_4. where Y is the predicted response. Model validation was verified as the actual and predicted values of 1789.46 mg/l, 1790 mg/l, respectively with productivity of 37.29 $mgl^{-1}h^{-1}$ and productivity yield of 596.6 (mg/g) (as illustrated in **Table 6**).

3.4 Gas chromatography analysis of total PUFAs produced by *Ps. fluorescens* NBRC11640

Fatty acids (FAs) composition of microbial oil produced by *Ps. fluorescence* is presented in **(Fig 4)** and **(Table 7)** Data obtained reveal that, saturated fatty acids (SFAs) represented more than one-fourth (27.55%) of FAs profile. Short chain FAs (C4:0- C10) were not found which was expected. SFAs group started from myristic acid (C14:0) and up to Behenic acid (C22:0), where the ratios of these two fatty acids represent 6.67 and 2.10 % of the total FAs, respectively. Palmitic acid was the highest (13.06 %) among all SFAs detected. Moreover, monounsaturated fatty acids (MUSFAs) were the lowest group (23.02 %) of FAs profile. Considering the microbial oil source, oleic acid (C18:1) was the dominant MUSFA followed by palmitoleic acid (C16:1, 5.92 %). The major group in the chromatogram **(Fig 4)** was polyunsaturated fatty acids (PUFAs) representing 47.83% of the total microbial oil FAs content. α-Linolenic acid (ALA, C18:3 ω-3) and linoleic acid (LA, C18:2 ω-6 cis) were the most dominant. ALA is a plant based essential ω-3 PUFAs that must be obtained through the diet. Nuts, seeds, and vegetable oils (flaxseed, canola, and soybean) contain appreciable amounts of ALA, and are sometimes used as a source for it (Iafelice et al 2008). ALA has proven essential for normal health, especially brain development and function. It has demonstrated also to be a potential nutraceutical for protecting the brain from stroke, characterized by its pleiotropic effects in neuroprotection, vasodilation of brain arteries, and neuroplasticity. Additionally, ALA can decrease low density lipoprotein (LDL) in the plasma that is correlated with the risk of developing atherosclerosis (Blondeau et al 2015). The data indicates that, the microbial oil can be considered as a good source for ALA instead of vegetable oils or nuts.



A. B. Olive cake and yeast extract

B. Olive cake and KH_2PO_4

Fig 3A-B. The two-dimensional contour plots and three-dimensional response surface plots showing the effect of olive cake and media components interactions on PUFAs production by *Pseudomonas fluorescens* NBR14160

Table 5. Observed and predicted PUFAs values of Central Composite Design

Run order	Actual levels				
	Olive cake (A) (g/l)	Yeast extract (B) (g/l)	KH_2PO_4 (g/l)	Actual Value mg/l	Predicted Value mg/l
1	10.00	10.00	0.50	422	446.50
2	15.00	3.30	1.25	1900	1892.20
3	15.00	7.50	1.25	1918	1789.46
4	15.00	7.50	0.00	658	686.68
5	15.00	7.50	1.25	1790	1789.46
6	10.00	5.00	2.00	648	702.86
7	23.41	7.50	1.25	900	1124.24
8	6.59	7.50	1.25	290	174.56
9	15.00	7.50	2.51	450	530.64
10	15.00	7.50	1.25	1790	1789.46
11	15.00	7.50	1.25	1790	1789.46
12	20.00	5.00	2.00	1642	1540.54
13	10.00	5.00	0.50	560	623.32
14	15.00	7.50	1.25	1790	1789.46
15	15.00	11.70	1.25	750	866.62
16	15.00	7.50	1.25	1678	1789.46
17	10.00	10.00	2.00	390	379.06
18	20.00	10.00	0.50	870	738.20
19	20.00	5.00	0.50	1700	1634.00
20	20.00	10.00	2.00	638	497.74

Table 6. Validation for the CCD model of medium components for optimized total PUFAs production by *Ps. Fluorescens*

Production parameters (and their levels)	Predicted value (mg/l)	Observed value (mg/l)	Biomass (g/l)	Productivity ($mgl^{-1}h^{-1}$)	Productivity yield coefficient relative to biomass (mg/g)
Olive cake (15)	1789.46	1790	3.00	37.29	596.60
Yeast extract (7.5)					
KH_2PO_4 (1.25)					

Productivity (P) = Amount of total lipid produced (**mgg^{-1}**) / fermentation time (h) = **$mgl^{-1}h^{-1}$**. PUFAs yield coefficient relative to biomass ($Y_{p/x}$) (mgg^{-1}) = Amount of PUFAs produced (mg/l) / amount of biomass (gl^{-1})

Table 7. Fatty acids composition of microbial oil

Fatty acids	Area %
Myristic acid C14:0	6.67
Palmitic acid C16:0	13.06
Stearic acid C18:0	3.20
Arachidic acid C20:0	2.52
Behenic acid C22:0	2.10
Total saturated fatty acids	**27.55**
Myristoleic acid C14:1	0.47
Palmitoleic acid C16:1	5.92
Oleic acid C18:1n9c	16.10
Elaidic acid C18:1n9t	0.53
Total monounsaturated fatty acids	**23.02**
Linolelaidic acid C18:2n9t	0.02
Linoleic acid C18:2n6c	13.11
α-Linolenic acid C18:3n3	19.60
Arachidonic acid C20:4nc	6.02
Eicosapentanoic acid EPA, C20:5	4.76
Docosahexaenoic acid DHA, C22:6	4.32
Total polyunsaturated fatty acids	**47.83**

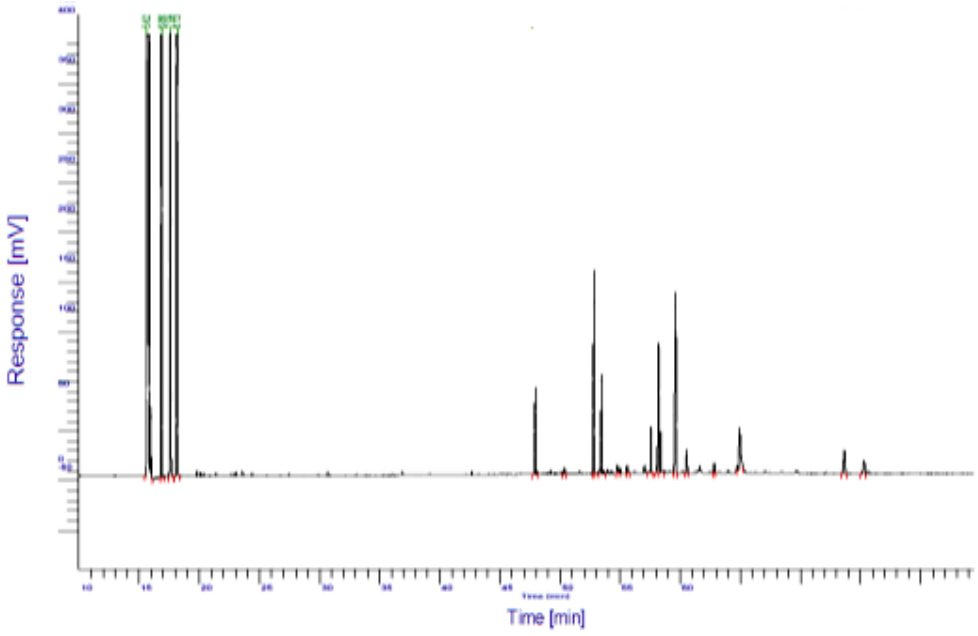


Fig 4. Chromatographic profile of compounds in microbial oil

3.5 Particle size distribution of microcapsules particles

The intensity of particle size distribution for microcapsules is given in (**Fig 5, A and B). (Fig 5, A)** shows the pattern of particle size intensity obtained from the basic suspension (1mg + 10 ml distilled water). As seen, 3 different size groups of particles were observed in the tested suspension. The first group has an average particle size of 5515 nm with an intensity 22.1%, while the second and third groups have particles with smaller sizes (785.6 and 212.1 nm) representing 51.9% and 26.1 of the total particle size, respectively. The presence of 3 particle size groups could be referred, according to Onsaard et al (2013) and Liu et al, (2020), to the multiple light scattering effect of the droplets (particles) in undiluted samples. Microcapsules containing particles with large diameters tend to sediment and segregate according to Stocks low (Liu et al, 2020). For a proper measurement of particle size distribution, emulsions should be diluted to a concentration < 0.005% (Gbassi et al, 2012 and Onsaard et al, 2013). The particle size distribution of the diluted microcapsules is given in **(Fig 5-b).** The peak of particle size was 671.4 nm with a poly dispersity index (Pdi) of 0.611. These average particle size agree with those reported by Agustinisari et al 2020 (498-834 nm). The moderate Pdi value of 0.611 indicates stability of the obtained emulsion due to the presence of whey protein concentrate glycated with maltodextrin to prevent the thermal denaturation of whey protein, (Sonu et al 2018). It should be mentioned that drying of encapsulated materials produces particles with larger hydrodynamic diameters.

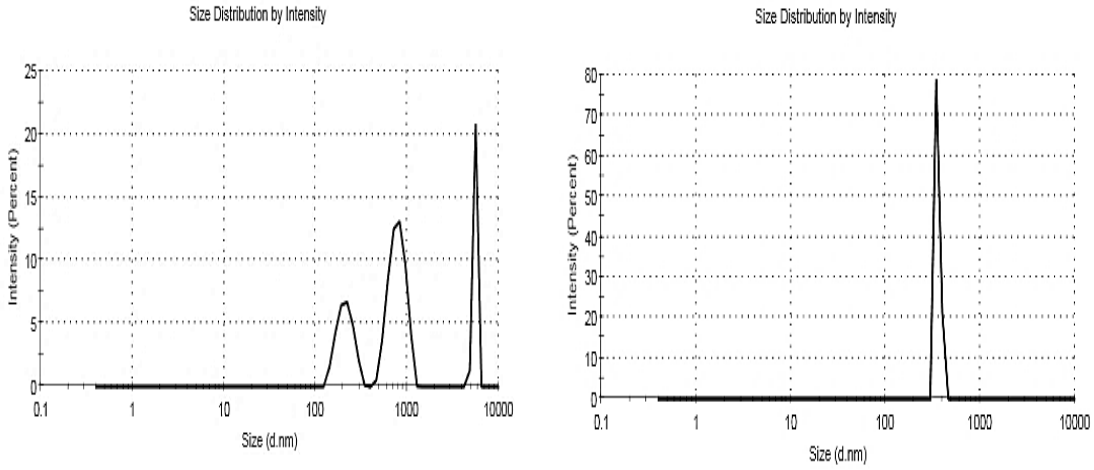
The pattern of size distribution by number of particles **(Fig 6)** did not differ from that obtained by intensity in **Fig 5.** The data obtained in **Fig 6, A** show that the number of large particles represent only 0.2% of the total particles in the tested emulsion, while the particles with size of 719.7 and 199.4 nm represents 6.7 and 92.1 % of the particle number in the suspension, which ensure their stability, since the majority of the particles are those of small size (199.4 nm). Results of diluted samples **(Fig 6, B)** showed that the suspension has one group of particles with average size of 667.1 nm and a narrow distribution index of 0.412, which assist the results presented in **Fig 5.**

3.6 Zeta potential of microcapsules

Zeta potential measures the net charge on the outer layer (surface) of particles in relation to the charges of ions present in the same electric field. In particles of microcapsules, the zeta potential measures the charges on the outer surface of whey protein concentrate layer in relation to the electric field of the surrounding electrolyte.

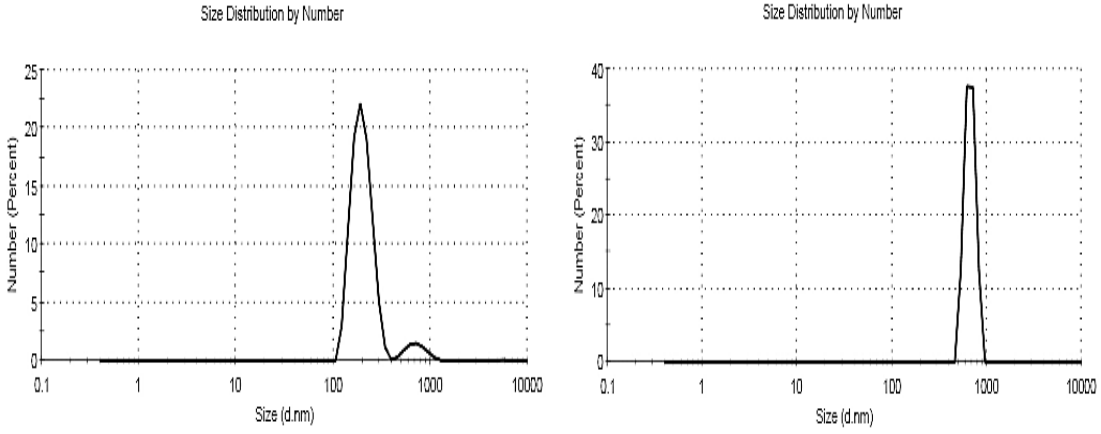
Fig 7 shows the potential of charge distribution on the surface of the suspension particles. As seen, the microcapsules particles showed a negative zeta potential of -37.6 mv in the diluted samples, which is close to the -42 mv value for oil emulsions stabilized by whey protein, maltodextrin and K-carrageenan (Onsaard et al 2013). According to Sonu et al, (2018) conjugation of whey proteins alters the distribution of protein surface charge, while glycation of whey protein with maltodextrin lead to reduction in lysine on protein surface, leading to shifting the isoelectric point towards lower pH values, which results in lower magnitude of zeta potential.

According to Liu et al (2020), suspensions with zeta potential above -30 mv are considered stable for long time. The higher values of zeta potential are signs for decreased attraction between particles and increased repulsion force. It is also worthy to mention that whey protein itself show positive zeta potential value between pH 1 and pH 5, then it shifted to negative values at higher pH values.



(A) **(B)**

Fig (5A-B) Particle size distribution by intensity for tested microcapsules.
A: Undiluted samples, B: diluted samples



(A) **(B)**

Fig (6A-B) Particle size distribution by number for tested microcapsules.
A: Undiluted samples, B: diluted samples

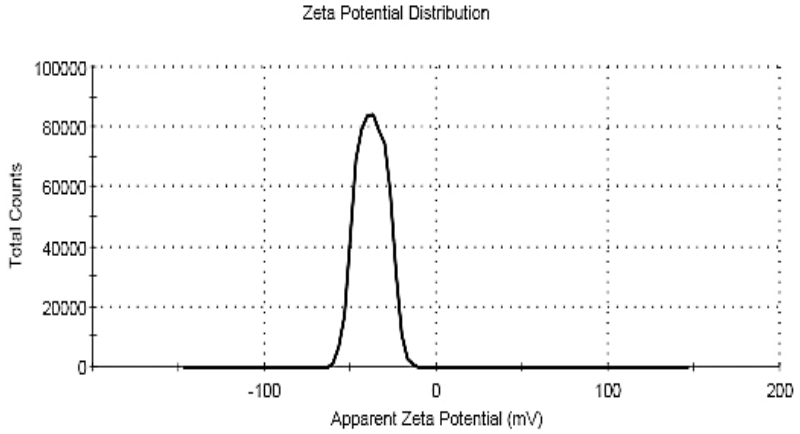


Fig 7 Zeta potential of tested microcapsules

3.7 Antioxidative activity of Kareish cheese samples

Fig 8 elucidates the antioxidative activity of tested Kareish cheese samples. As seen, antioxidative activity of control samples was 30.14%. Incorporation of 0.5% microcapsules containing PUFAs did increase the antioxidative activity to 38.13%. Further increasing the addition ratio of microcapsules to 1% and 1.5% raised the antioxidative values to 42.19% and 48.11%, respectively. Presence of unsaturated fatty acids in cheese samples made it more stable for oxidation compared to the control. The role of encapsulated polyunsaturated fatty acids in enhancing the antioxidative activity of soft cheese was discussed by Khalifa et al (2017), Rahman et al (2017) and Park et al (2018). They reported that PUFAs from plant sources (Peanut oil, olein fraction of chia and oleogel) did enhance the antioxidative stability of soft cheese and ice cream through maintaining the peroxide value (PV) lower than allowable limit of 10 meq O_2 /Kg. The antioxidative activity of the tested cheese samples were decreased by increasing the cold storage period to 28 days. The values for antioxidative activity were reduced to the values of 16.05, 30.12, 35.04 and 38.28%, respectively for T_0, T_1, T_2 and T_3. According to Rahman et al (2017), increasing the level of PUFAs lead to decline in flavor score of ice cream due to the generation of peroxides during the storage period, which agree with the results of sensory evaluation reported in the present work.

3.8 Texture profile analysis of kareish cheese

Fig 9 represents the textural behavior of Kareish cheese during the two successive compression cycles. **Table 8** shows the obtained texture profile parameters of kareish cheese samples. The evaluation includes the parameters Hardness, Adhesiveness, Cohesiveness, Springiness, Gumminess, Chewiness and elastic module. The fresh control samples showed hardness value of 1.9 N, which was increased to 2.13, 2.56 and 4.07 N by increasing the level of added microcapsules to 0.5, 1 and 1.5 %, respectively.

Similarly, the work done during the first compression cycle recorded 6.33, 8.17, 8.27 and 14.15 mj as the addition level of microcapsules was increased from 0 % to 0.5, 1 and 1.5 %, respectively.

The increase in hardness is justified, since whey protein concentration and maltodextrin supports the consistency and gel net of cheese. It should be mentioned that the values of hardness depend, beside production conditions and

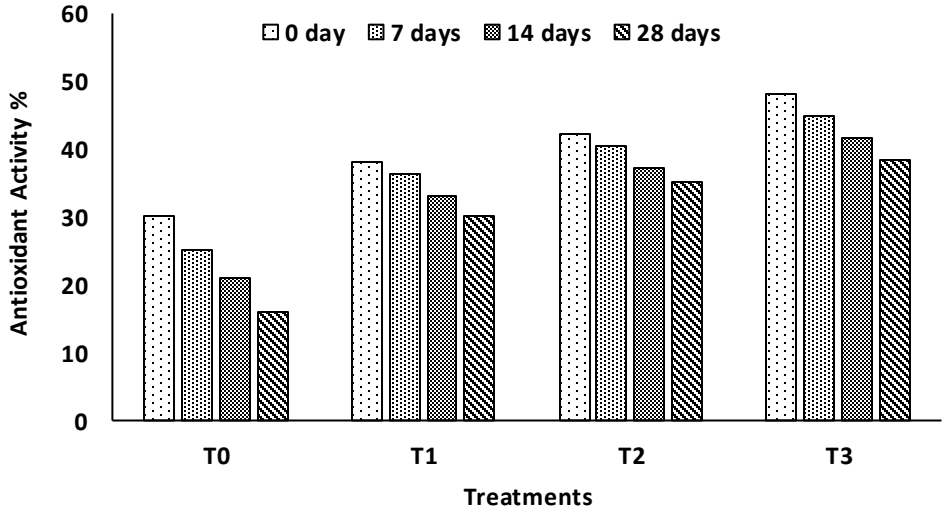
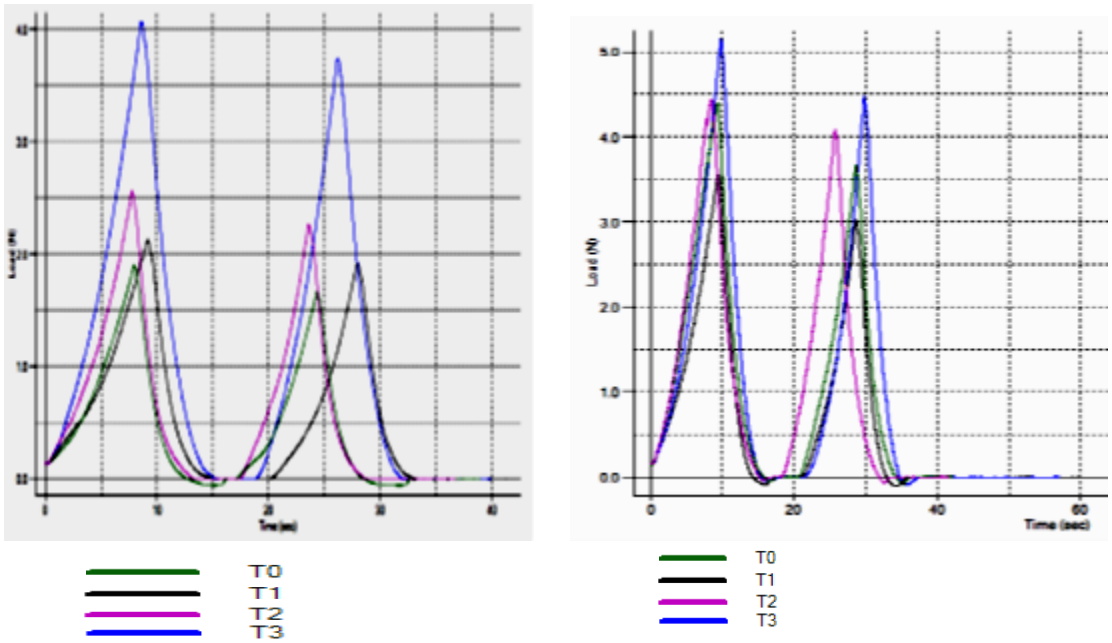


Fig 8. Antioxidative activity of tested Kareish cheese samples
T0: control, T1: 0.5%, T2: 1%, T3: 1.5% (fatty acids microcapsules)



(A) (B)

Fig 9. Texture profile pattern of tested Kareish cheese samples.
A: fresh, B: 28 days cold storage

Table 8. TPA Parameters of Kareish cheese treatments

Treatment	Storage day	Hardness (N)	Adhesiveness (mJ)	Cohesiveness (Ratio)	Springiness (mm)	Gumminess (N)	Chewiness (mJ)
T_0	Fresh	1.900^{Cd}	0.271^{Bb}	0.68^{Ba}	5.70^{Bc}	1.297^{Bc}	7.41^{Cd}
	7	3.732^{Cc}	0.275^{Ba}	0.60^{Bc}	5.36^{Bd}	2.236^{Bb}	12.59^{Cc}
	14	4.062^{Cb}	0.147^{Ba}	0.67^{Bb}	6.96^{Bb}	2.724^{Ba}	18.96^{Cb}
	28	4.399^{Ca}	0.172^{Bb}	0.61^{Bbc}	7.16^{Ba}	2.687^{Ba}	19.24^{Ca}
T_1	Fresh	2.128^{Dd}	0.169^{Ab}	0.65^{Ba}	6.34^{Cc}	1.393^{Cc}	8.96^{Dd}
	7	2.698^{Dc}	0.227^{Aa}	0.61^{Bc}	4.94^{Cd}	1.646^{Cb}	8.12^{Dc}
	14	3.252^{Db}	0.295^{Aa}	0.64^{Bb}	6.33^{Cb}	2.066^{Ca}	12.08^{Db}
	28	3.560^{Da}	0.252^{Ab}	0.62^{Bbc}	6.62^{Ca}	2.197^{Ca}	14.55^{Da}
T_2	Fresh	2.559^{Bd}	0.157^{Db}	0.65^{Aa}	5.38^{Dc}	1.674^{Bc}	9.00^{Bd}
	7	3.058^{Bc}	0.127^{Da}	0.68^{Ac}	5.07^{Dd}	2.065^{Bb}	10.47^{Bc}
	14	4.335^{Bb}	0.143^{Da}	0.68^{Ab}	6.39^{Db}	2.965^{Ba}	18.96^{Bb}
	28	4.440^{Ba}	0.150^{Db}	0.72^{Abc}	6.70^{Da}	3.185^{Ba}	21.35^{Ba}
T_3	Fresh	4.069^{Ad}	0.121^{Cb}	0.75^{Aa}	6.48^{Ac}	3.064^{Ac}	19.85^{Ad}
	7	4.875^{Ac}	0.187^{Ca}	0.59^{Ac}	5.52^{Ad}	2.858^{Ab}	15.78^{Ac}
	14	4.650^{Ab}	0.225^{Ca}	0.64^{Ab}	6.77^{Ab}	2.954^{Aa}	19.99^{Ab}
	28	5.156^{Aa}	0.157^{Cb}	0.66^{Abc}	7.15^{Aa}	3.397^{Aa}	24.30^{Aa}

A,B,C,D Means in same at each parameter of treatment with different uppercase letters differed significantly ($p < 0.05$) a,b,c,d Means in same at each parameter of storage with different lowercase letters differed significantly ($p < 0.05$)

gel structure of cheese, on TPA test conditions such as type and diameter of the used compression device, deformation ratio, applied cell load and cross head speed during the TPA test. Therefore, hardness values reported in the literature ranges between 5.5 N, to 91 N (Hussein and Shalaby, 2014, Dimitreli et al 2017, Abbas et al 2017, Metry et al 2018, as well as El-Sayed and El-Sayed, 2020). The lack of test details complicates the comparison of the obtained results with published data.

On contrary to hardness, Adhesiveness values of kareish cheese (in mj) were decreased from 0.271 for control sample to only 0.121 for cheese sample containing 1.5 % of the added microcapsules.

The Cohesiveness values of the tested kareish cheese samples was in the range of 0.68 to 0.75, being higher than those reported in the literatures (0.22 to 0.63) due to the moderate deformation ratio (25 %), which maintain the cheese structure in the elastic deformation region. On the same manner, springiness (in mm), remained in the range of 5.38 to 6.48 mm for all fresh samples, being higher than those reported by the aforementioned researches (0.54 to 6.5 mm) due to the conditions that the TPA test was carried out in the elastic deformation region of the tested cheese samples. Gumminess and chewiness are two parameters related to the force and work required to bite and swallow the cheese samples. As seen in **Table 8**, gumminess value of the fresh control sample was 1.3 N and it was increased to the level of 3.1 N as the addition of microcapsules reached 1.5 %. Gumminess value was increased by almost 183% as the addition level was increased from 1 % to 1.5 %. Chewiness values of fresh samples recorded 7.41, 8.96 and 9 mj, respectively as the addition level was raised from 0 to 0.5 and 1 %. Increasing the microcapsule level to 1.5 % dramatically increased the chewiness energy to 19.85 mj, which makes an increase of more than 220 % in the work required during breaking and swallowing the cheese sample. Elastic modules were calculated as the ratio (slope) of hardness (N) and deformation (mm) and recorded 0.25, 0.24, 0.35 and 0.50 (N/mm), respectively for T0, T1, T2 and T3. An increase in elastic module means, from mechanical point of view, a change from elastic to stiff and brittle type of

structure. Storage of the tested cheese sample for 28 days led to increase in hardness, springiness, gumminess and chewiness values and decrease in cohesiveness ratio of the tested samples, which comply with loss in moisture (2.5%, data not tabulated). The work required to compress the stored cheese samples was increased to the level of 18.82, 14.02, 16.53 and 19.56 mj for T0, T1, T2 and T3 samples respectively, where T1 treatment (0.5 % microcapsules) showed the lowest work (14.02 mj) for compression. Also T1 showed the lowest elastic module (0.493 N/ mm) at the end of storage period, while the other cheese samples, including the control, showed elastic module close or higher than 0.5 N/mm, which prove the stiffness and the brittleness of these samples at the end of storage period.

3.9 Sensory evaluation of Kareish cheese

Table 9 shows the scores recorded for the panel test of the prepared kareish cheese samples. As seen, the cheese samples of treatment T1 (0.5% microcapsules) recorded the highest scores for flavor, appearance and total scores, while there was no significant difference in the body and texture between the treatments T1, T2 and T3 but they all differed from the control sample (T0). Although all sensory parameters recorded lower values during the 28 days of storage, the treatment (T1) obtained significantly the highest total scores among the other tested cheese samples. The sensory judgment of the cheese treatment (T1) confirms the superior textural parameters of the same sample mentioned in **Table 8**. Therefore, the cheese sample (T1) could be considered as the best treatment.

Table 9. Sensory evaluation of Kareish cheese treatments with add different levels of microcapsules

Treatments	Storage period (days)	Flavor (50)	Body and Texture (35)	Appearance (15)	Total scores (100)
T_0	Fresh	47^{Aa}	33^{Ba}	13^{Ba}	93^{Ba}
	14	46^{Ab}	30^{Bb}	12^{Ba}	88^{Bb}
	28	42^{Ac}	28^{Bc}	10^{Bb}	80^{Bc}
	Fresh	49^{Aa}	34^{Aba}	14^{Aa}	97^{Aa}
T_1	14	47^{Ab}	32^{Abb}	13^{Aa}	93^{Ab}
	28	45^{Ac}	30^{ABc}	12^{Ab}	88^{Ac}
	Fresh	45^{Ba}	35^{Aa}	12^{Ba}	92^{Ba}
	14	44^{Bb}	33^{Ab}	12^{Ba}	89^{Bb}
T_2	28	41^{Bc}	29^{Ac}	11^{Bb}	81^{Bc}
	Fresh	43^{Ca}	35^{Aba}	12^{Ba}	90^{Ca}
	14	40^{Cb}	33^{Abb}	12^{Ba}	85^{Cb}
	28	39^{Cc}	29^{ABc}	10^{Bb}	78^{Cc}
T_3	Fresh	47^{Aa}	33^{Ba}	13^{Ba}	93^{Ba}
	14	46^{Ab}	30^{Bb}	12^{Ba}	88^{Bb}
	28	42^{Ac}	28^{Bc}	10^{Bb}	80^{Bc}
	Fresh	49^{Aa}	34^{Aba}	14^{Aa}	97^{Aa}

A,B,C Means in same at each parameter of treatment with different uppercase letters differed significantly ($p < 0.05$) a,b,c Means in same at each parameter of storage with different lowercase letters differed significantly ($p < 0.05$)

4 Conclusion

In this study, response surface methodology, as statistical approach, showed to be more adequate and efficient for the optimization PUFAs production from Olive cake waste by *Pseudomonas fluorescence*. Experimental conditions adopted in this work increased the PUFAs production from 22 mg/l in PBD to reach 1789.46 mg/l (i.e. 81.3 times increase). GC analysis of the obtained PUFAs showed the presence of more than 47% polyunsaturated fatty acids including oleic acid (C18:1), palmitoleic acid (C16:1), α-Linolenic acid (ALA, C18:3 ω-3) and linoleic acid (LA, C18:2 ω-6 cis). Application of PUFAs in Kareish cheese led to a significant enhancement in all cheese properties.

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