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Using Cheese whey for the Production of Carotenoids, Ergosterol and Novel Functional Foods of Industrial interest though a series of Optimized Bio- and Chemical- Processes

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Abstract*—The increasing industrial demand for the production of innovative functional food (lactose free products) as well as bio-compounds with nutritional value (such as carotenoids and other metabolites like ergosterol), that could find several applications in industrial food sector (Research and Development department) has increased interest regarding their potential production (employing applied biochemistry and biotechnology principles in their optimized production processes). Reduction of total production costs has encouraged the usage of low-cost or negative valued agroindustrial by-products or waste streams to industrial food sector. Cheese whey (after being deproteinized) was treated, either with acid (HCL) or commercial β-galactosidase (from Aspergillus oryzae), aiming at hydrolyzing initial lactose contained in a unique mixture of cheese whey, delivered by different cheese whey making processes, from Aegean islands (Greece). Regarding HCL (37% vv^{-1}) catalyzed hydrolysis of unique Aegean islands (Greece) delivered cheese whey, the highest glucose concentration of 3873.66 mgL^{-1} achieved after 120 min of reaction, at 100 ^oC at a pH range ranking from 1 to 1.1. As for enzymatic catalyzed hydrolysis process of cheese whey, using β-galactosidase (from Aspergillus oryzae), maximum production of 18.78 gL^{-1} glucose, achieved at 55 ^oC and pH= 5, after 12 hours of enzymatic hydrolysis (when the initial cheese whey concentration and initial enzymatic activity was 1200 gL^{-1} and 9 UmL^{-1}, respectively). Evaluating potential usage of cheese whey hydrolysates in industrial food sector, it was concluded that a 6h enzymatic process was adequate for the production of glucose-rich streams, that could find several applications toward the production of novel functional foods (free of lactose or low lactose content), improving their sensorial and technological properties (while addressed to those suffering from lactose intolerance). Regarding bioreactor fed-batch bioconversions by Rhodotorula glutinis, using enzymatically prepared cheese whey hydrolysates as generic feedstock (with initial glucose concentration of 18 g L^{-1}), the highest production of total carotenoids, ergosterol and total dry weight achieved was 127.3 ± 0.41 μg g^{-1} (or 2023.03 ± 0.41 μgL^{-1}), 170.78 ± 0.38 μg g^{-1} (or 2703.917 ± 4.37 μgL^{-1}), respectively. The flow aeration rate was maintained at 2vvm.The pH value was regulated by using 5M NaOH and 10% (vv^{-1}) H_2SO_4, at optimum range (6.2-6.5). The dissolved oxygen concentration at the bioreactor, was regulated at 30% of saturation. Cheese whey, a by-product of cheesemaking process, could form an ideal feedstock through the designing and development of optimized series of bioprocesses leading to the development of a novel biorefinery that could produce several value-added products with high nutritional value and several commercial marketed outputs.*

Keywords — cheese whey, applied biosciences, biotechnology, carotenoids, ergosterol, optimized processes, research and development.

I. INTRODUCTION

Carotenoids represent one of the most important classes of components in food, effecting their acceptability, used widely in industrial food sector as coloring agents or additives (their color vary from the yellow to red range), affecting the major sensory characteristics (color and acceptability) of foods. Carotenoids are lipid-soluble

pigments (the majority of them are C_{40} terpenoids), acting as membrane-protective antioxidants scavenging O_2 and peroxyl radicals. Thus, their antioxidant activity could be attributed to their structure. Interest in carotenoid production has been increased lately, owning to their related beneficial effects in human health as well as the growth of certain areas of industrial food sector such as agriculture, aquaculture and poultry industry (Dimou *et al*., 2019a; 2019b). Carotenoids are used as coloring additives in food systems, while enriching them with provitamin-A, often increasing their antioxidant activity (Dimou *et al*., 2019a). These bioactive compounds have been used as additives for the production of a wide range of food (such as cooked sausages, soft drinks, baked goods), pharmaceutical and cosmetic formulations. Besides, humans do not synthesize carotenoids de novo but take them in the diet, using them as precursors for the production of retinoids such as vitamin A (Dimou *et al*., 2019b). In fact, carotenoids are bioactive phytochemicals that have been credited of reducing risks of development of degenerative diseases such as cancer, cardiovascular diseases, macular degeneration and cataract (Dimou *et al*., 2017a). These bioactive compounds could be used for the production of several novel functional foods and nutrient supplements. It is expected that the growing demand for these healthy value-added products will boost carotenoid bioprocessing as a fundamental player to meet the requirements of consumers and industry. Carotenoids are primarily produced by filamentous fungi and yeasts as well as by some species of bacteria and algae (Dimou *et al*.,2019c; 2019d). Among microbial sources used for the production of biomas -enriched metabolites such as carotenoids and ergosterol of commercial interest, *Rhodotorula glutinis* is one of the most well-known species (Martinez *et al*., 2009; Dimou *et al*., 2019c; Koutelidakis *et al*.**,** 2019). Ergosterol is a biological precursor of vitamin D_2, produced through the implementation of suitable bioprocessing system, using yeasts as microbial strains. Ergosterol exists in yeast cell wall membranes and mitochondria. It has been reported, that hyphomycetes and ascomycetes, contain ergosterol in variant concentrations ranging from 2.3 to 11.9 μg of ergosterol/mg of dry weight. Also, the ergosterol content for *Cladosporium* sp, *Candida* sp, and *Alternaria* sp, varies from 0.4 to 14.3 μg/mg according to Pasanen *et al*. (1999). Bioprocessing derived ergosterol could be used as a dietary supplement and/ or food additive, for the production of ergosterol rich functional products (Corrêa *et al*., 2018). Research in that area is very limited (Dimou *et al*., 2019c; Koutelidakis *et al*., 2019). Trying to improve the yield of metabolite products and subsequently decrease the cost of bioprocessing, optimization of culture conditions including the usage of cheap agroindustrial byproducts or waste streams as nutrient supplements, form a current emerging tendency toward the production of bioderived value added products of high nutritional value and industrial interest (Dimou *et al*., 2015; 2016a; 2016b; 2016c; 2017a; 2017b; 2019a; 2019b; 2019c). Several agroindustrial by-product or waste streams from industrial food sector such as sugarcane, sugar beet molasses, cheese whey, hydrolyzed beans, corn meal, corn steep liquor, soybean oil, wine lees, grape pomace have been used for the production of value-added products (Dimou *et al*., 2015; 2016; 2017a; 2017b; 2019a; 2019b; 2019c; Koutelidakis *et al*., 2019; Kopsahelis *et al*., 2018).

Indeed, such media are very complex and further pretreatment steps may be needed so as to increase the bioavailability of nutrients to microorganisms (Dimou *et al*., 2015; 2016; 2017a; 2017b; 2019a; 2019b; 2019c. Cheese whey, a byproduct stream derived from cheese making process, constitutes a promising raw material for bioprocessing, since nearly 55% of milk nutrients (proteins, lactose and minerals salts) remain in that fraction, during cheese processing. Worldwide production of cheese whey is approximately 145×10^9 kg $year^{-1}$ (Dimou *et al*., 2019a). To the best of our knowledge there is no literature cited publication studying potential concurrent production of carotenoids, ergosterol and other nutrient supplements rich in glucose, using cheese whey, as low cost agroindustrial substrate.

The objective of this study was the evaluation of potential production of bio-carotenoids, ergosterol and other value-added functional feedstocks, with nutritional value, using cheese whey as raw material, through the development of bio-science based processes. Another goal of this research was the optimization of processes (enzymatic or acid catalyzed) leading to concurrent production of novel nutrient supplements rich in glucose, which could then be further exploited toward the production of either innovative functional foods or as a generic feedstock leading to the production of bio-carotenoids and ergosterol, using *Rhodotorula glutinis* strain.

II. MATERIALS AND METHODS

2.1 Substrates

Cheese whey effluents derived from different cheese production processes leading to the production of the very well-known Greek types of cheese: "Kalathaki", "Anthotiro", "Feta'' and "Melixloro", were obtained from local dairy plants from Aegean islands (mainly from Lemnos). For all the experiments performed, cheese whey used was a mixture, containing: 40 % vv^{-1} cheese whey derived from "Kalathaki" production; 10 % vv^{-1} cheese whey formulated during "Anthotiro" production; 40 % v v^{-1} cheese whey derived from "Feta" production and 10 % v v^{-1} cheese whey derived from "Melixloro" production.

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The cheese whey mixture content was: 95% water; 65 g L^{-1} dry weight; 25 gl^{-1} ash; 38 gL^{-1} lactose; 0.3 gL^{-1} glucose; 58 mgL^{-1} phosphorus; 1.8 gL^{-1} soluble proteins and 0.1% total nitrogen. Cheese whey was evaluated as raw material for the bioproduction of value added products of nutritional value that could be used either as glucose rich streams free of lactose towards the production of novel functional foods or as a generic feedstock for the production of biomass-enriched metabolites (carotenoids and ergosterol) of commercial food and nutrition industrial interest.

2.2 De-proteinisation of cheese whey

De-proteinisation of cheese whey performed in an autoclave at 121ºC for 20min. After coagulation and precipitation of denaturated proteins the sample was centrifuged at 1000×g and 20ºC for 15 min. The supernatant was transferred to Erlenmeyer flasks and immediately used for hydrolysis and media formulation. The sediment, consisted of precipitated proteins, were stored at -40 ^{0}C.

2.3 Production of hydrolysates: Acid hydrolysis of cheese whey

The acid hydrolysis of cheese whey performed in 2000 mL Erlenmeyer flasks, placed in a shaking water bath (TC-202P Circulating, Ametek, Brookfield). Three sets of experiments were carried out to evaluate the effect of a) pH (1-5), b) reaction time (10-120 min), c) temperature (50-120 ºC) during acid hydrolysis of cheese whey, catalyzed by 1N HCl (37 %, Sigma Aldrich). Mixing of the suspension took place using magnetic stirrers. Samples were collected at random intervals. Remaining solids were separated by centrifugation (10min, 3000g). The supernatant was used for the analyses of glucose concentration. Hydrolysis yield was expressed as the percentage of lactose concentration of cheese whey to final glucose concentration of cheese hydrolysate. After the end of acid hydrolysis, cheese whey hydrolysates were pretreated, as described previously Dimou *et al*. (2012a; 2012b) so as to formulate a suitable nutritional supplement for *R. glutinis* bioconversions. All the aforementioned experiments were performed in triplicate.

2.4 Production of hydrolysates: Enzymatic hydrolysis of cheese whey

The enzymatic hydrolysis of cheese whey performed in 2000 mL Erlenmeyer flasks, by *Aspergillus oryzae* β-galactosidase of 3 UmL^{-1}, 6 UmL^{-1}, and 9 UmL^{-1} enzymatic activity, in orbital rotation shaker (MaxQ 4000 Benchtop, ThermoFisher Scientific), at 160 rpm. The effect of a) temperature (10°C and 55°C), b) reaction time (0h-12h), c) enzymatic activity (3 UmL^{-1}, 6 UmL^{-1}, and 9 UmL^{-1}), d) initial cheese whey concentration (500 gL^{-1}, 800 gL^{-1}, 1200 gL^{-1}) and e) pH (1-7) regarding glucose production was studied. The enzymatic concentrations of 3 UmL^{-1}, 6 UmL^{-1}, and 9 UmL^{-1} corresponded to 0.1 gL^{-1}, 0.2 gL^{-1}, and 0.3 gL^{-1} of *A.oryzae* β-galactosidase, respectively. At random intervals samples were taken and simultaneously submitted to heating at 100°C for 10 min, so as to inactivate the enzyme. After the end of acid hydrolysis, cheese whey hydrolysates were pretreated, as described previously Dimou *et al*. (2012b; 2015; 2016a; 2019a; 2019c) and Koutelidakis *et al*. (2019) so as to formulate a suitable nutritional supplement for *R. glutinis* bioconversions. The treated samples were stored at -40ºC for further determination of glucose. All the experiments were performed in triplicate.

2.5 Micro-organism and cultivation conditions

A strain of *Rhodotorula glutinis* CCY 20-2-26, donated from culture collection, Bratislava, Slovakia was evaluated for potential bioproduction of carotenoids and ergosterol. Initially the lyophilized culture was hydrated in YM medium (yeast/malt extract: 3 gL^{-1} yeast extract, 3 gL^{-1} malt extract, 5 gL^{-1} peptone and 18 gL^{-1} glucose) at 28ºC for 72h. Then the culture was transferred to slant tubes containing YMA medium (yeast/malt extract agar: this medium contains the same composition as YM medium including also 20 gL^{-1} agar) and incubated at 26 ºC for 72h. After growth the slants were kept at 4 ^{0}C and sub-cultured every 2 months. Stock cultures were also preserved at -80 ºC, containing 200 gL^{-1} glycerol. Inocula obtained from cultures grown on YM slants at 28 ºC for 24 hr. A loop of the yeast cells was transferred to 250 mL Erlenmeyer flasks containing the production medium and incubated at 26 ºC for 40 h, in a rotary shaking incubator (Lab-Line Incubator-Shaker) at 160 rpm.

2.6 Production of value-added products: carotenoids and ergosterol

Prior to each cultivation cheese whey and cheese whey hydrolysates were treated as described earlier (Dimou *et al*.; 2015; 2016a; 2018). Yeast fermentations carried out in 250 mL Erlenmeyer flasks (50 mL broth) placed in a rotary shaker and agitated at 160 rpm, using 1mL of preculture standard medium as inoculum. Crude enzymatic prepared hydrolysates, of variant glucose concentration (3.5-18 gL^{-1}), used as nutrient supplements, aiming at evaluating potential concurrent bio-carotenoids and ergosterol production. Fed-batch bioreactor bio-conversions performed as described earlier, according to Dimou *et al*., (2015; 2016a; 2018). Inoculations took place using a 10 % (vv^{-1}), preculture as inoculum. The flow aeration rate was maintained at 2 vvm. pH-value was regulated by using 5M NaOH and 10% (vv^{-1}) H_2SO_4, at

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optimum pH range (6.2 - 6.5). The dissolved oxygen concentration at the bioreactor, was regulated at 30% of saturation, by controlling the agitation speed at the range of 200-500 rpm. Concentrated cheese whey hydrolysates, containing a variant concentration of glucose ranking from 15 to 20 gL^{-1}, used for feeding. The salts solution as well as yeast extract concentrations, used as bioprocessing feedstock standardized mixture contained: 0.15g L^{-1} $MgSO_4 \cdot 7H_2O$, 5 gL^{-1} K_2HPO_4, 5% NaCl and 5 gL^{-1} yeast extract. All yeast fermentations were carried out in triplicate. Samples of 5mL were taken at random intervals.

2.7 Analytical methods

Glucose concentration measured according to the dinitrosalicylic acid (DNS) method Miller, 1959. Ergosterol and carotenoids were extracted and assayed as previously described by Dimou *et al*. (2019c) and Koutelidakis *et al*. (2019). β-galactosidase activities of the commercial enzymes were assayed according to Cruz *et al*.,1999 and Koutelidakis *et al*. (2019)**.** The total cell dry weight was determined according to Dimou *et al*. (2015; 2016).

III. RESULTS AND DISCUSSION

3.1 Acid catalyzed hydrolysis of cheese whey

The highest glucose concentration of 3873.66 ± 2.239 mgL^{-1} was obtained after 120 min of acid catalyzed (deproteinized) cheese whey hydrolysis, at 100 ^oC, at pH values around 1. As it can be seen in Table-1, as pH value of cheese whey medium increase from 1 to 5, final produced glucose concentration is decreased. On the contrary, as reaction time increases from 10 to 120 min, final produced glucose concentration is increased, reaching its maximum accomplished concentration of 3873.66 ± 2.239 mgL^{-1}, after 2h of reaction (Table 1). Further increase in reaction time (120 min), did not affect final glucose concentration. This finding could be possibly attributed to parallel reactions that took place throughout cheese whey hydrolysis, such as Maillard and glucose degradation reactions.

Table.1: Acid hydrolysis of cheese whey: Glucose concentration throughout acid hydrolysis process at variant pH values (1-5) and reaction times (10-120 min), at 100 ^oC.

Time of reaction (min)	pH	Final glucose concentration* (mgL^{-1})
10	1	336.660 ± 0.778
10	2	235.780 ± 6.985
10	3	103.420 ± 3.549
10	4	092.540 ± 0.603
10	5	078.210 ± 1.983
30	1	726.620 ± 6.481
30	2	642.860 ± 1.801
30	3	378.730 ± 3.993
30	4	220.920 ± 1.604
30	5	180.820 ± 3.681
50	1	1900.28 ± 1.108
50	2	1370.67 ± 4.441
50	3	810.930 ± 4.147
50	4	412.020 ± 5.840
50	5	309.540 ± 2.033
80	1	3283.34 ± 1.230
80	2	2201.72 ± 1.730
80	3	1278.34 ± 9.575
80	4	641.270 ± 2.351
80	5	314.670 ± 2.534
120	1	3873.66 ± 2.239
120	2	2467.78 ± 6.179
120	3	1330.24 ± 2.788
120	4	683.670 ± 2.497
120	5	378.280 ± 3.613

*The results are expressed as the mean values of three replicates (±SD)

Glucose production throughout 120 min of cheese whey hydrolysis at optimum pH=1, at varying temperatures ranging from 50 ^oC to 120 ^oC are presented in Fig-1. It can be easily observed that increasing temperature of acid-catalyzed hydrolysis process from 50 ^oC to 100 ^oC, (maximum glucose produced after 120min of reaction Table-1) also final glucose concentration increased from 0.97 gL^{-1} to 3.872 gL^{-1}, implying that temperature is a very crucial parameter that affects lactose to glucose hydrolysis efficiency.

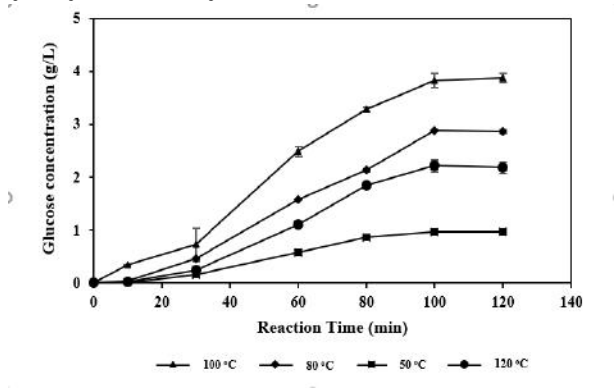

Fig.1: Acid hydrolysis of cheese whey during 120h of acid catalyzed process at 50 ^oC; 80 ^oC; 100 ^oC; 120 ^oC at optimum pH=1

*The results are expressed as the mean values of three replicates (±SD)

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On the other hand, further increase of the reaction temperature, above 100°C, did not positively affect glucose production in the final produced hydrolysate (Fig.1). It seems that during sugar acid hydrolysis at pH values lower than 2 and temperature above 100°C, furfural, formic acid, acetic acid and 5-hydroxymethylfurfural are produced, hindering hydrolysis efficiency. To the best of our knowledge, such an investigation, evaluating potential production of acid catalyzed cheese whey (derived from "Kalathaki", "Anthotiro", "Feta'' and "Melixloro" cheese production process, from Aegean islands) hydrolysates as well as optimization of this process has never before been published.

3.2 Hydrolysis of cheese whey via enzymatic hydrolysis

Hydrolysis of cheese whey using *Aspergillus oryzae* β-galactosidase, aiming at producing glucose rich streams as well as optimization of enzymatic process was studied. The effect of several parameters, affecting enzymatic process, : a) temperature (10 °C and 55 °C), b) reaction time (0h-12 h), c) initial enzymatic activity (3 UmL^{-1}, 6 UmL^{-1}, and 9 UmL^{-1}), d) initial cheese whey concentration (500 gL^{-1}, 800 gL^{-1}, 1200 gL^{-1}) and e) pH (1-7) were evaluated.

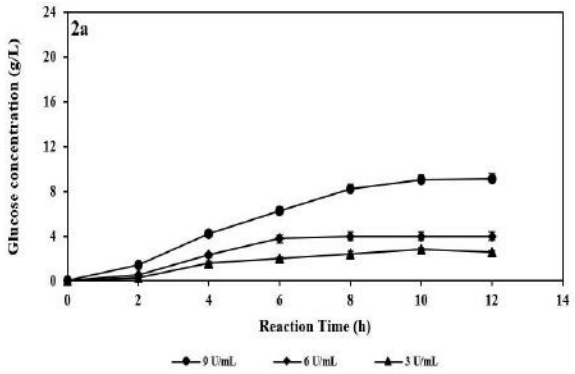

Fig.2: Enzymatic hydrolysis of cheese whey throughout 12h of reaction bioprocess, using A. oryzae derived β-galactosidase preparations of 3 UmL^{-1}, 6 UmL^{-1}, 9 UmL^{-1} at optimum pH (of 5) and initial cheese whey concentration (1200 gL^{-1}). Glucose production at (2a) 10°C (2b) 55°C.

*1 one unit of β-galactosidases activity (U) was defined as the amount of enzyme that liberates 1.0 μmole of o-nitrophenol per minute under assay conditions

*2: The results are expressed as the mean values of three replicates (±SD)

The effect of the temperature (10°C and 55°C), reaction time (0-12h), initial enzymatic activity of *A. oryzae* β-galactosidase (3 UmL^{-1}, 6 UmL^{-1}, and 9 UmL^{-1}), at optimum pH (approximately equal to 5) and initial cheese whey concentration (of 1200 gL^{-1}), are shown in Fig. 2. The main purpose regarding cheese whey hydrolysis experiments performed at low temperature (10°C), was the production of a novel glucose-rich stream using cheese whey as raw material without affecting its sensorial and nutritional characteristics. According to the results presented in Fig. 2 all the studied parameters affected the enzymatic process and thus, final glucose production and hydrolysis degree. Maximum production of (approximately) 18.78 gL^{-1} glucose (more specifically 18.777 ± 0.007 gL^{-1}), achieved at 55°C, pH around 5, after 12 hours of enzymatic hydrolysis of 1200 gL^{-1} cheese whey when the initial enzymatic activity of β-galactosidase was 9 UmL^{-1} (Fig-2b). It has been reported, that the enzymatic activity of *A. oryzae* enzymes (including β-galactosidase) may be influenced by multiple environmental factors, among which the most important are temperature, pH and initial enzymatic activity (Dimou *et al*., 2015). It seems that these factors possibly affect the tridimensional structure or the remaining protein conformation in the medium, affecting hydrolysis efficiency (Dimou *et al*., 2016; Jurado *et al*. 2004). Additionally, it could be concluded that the enzymatic activity directly affects lactose hydrolysis, throughout hydrolysis process. Increasing initial β-galactosidase enzymatic activity from 3 UmL^{-1} to 9 UmL^{-1}, final glucose concentration presented a two point four-fold increase, approximately (Fig.2b).

The effect of concentration on cheese whey hydrolysis yield or efficiency, at optimum conditions (initial enzymatic activity=9UmL^{-1}; T=55°C, pH=5, time=12hours), has been also studied.

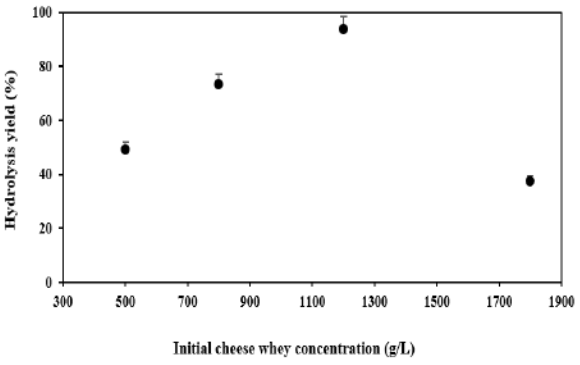

Fig.3: Effect of initial cheese whey concentration on hydrolysis yield (lactose to glucose conversion), at optimum conditions (9 UmL^{-1} enzymatic activity, reaction time=12h; T=55°C)

Fig.3 shows that the hydrolysis efficiency was maintained above 73% at initial cheese whey concentrations at the range of 800-1200 gL^{-1}, while it decreased significantly when using initial cheese whey concentration higher than 1200 gL^{-1} (e.g 37 % when initial cheese whey concentration was 1800 gL^{-1}). Similar trend has been

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previously, reported by Dimou *et al*. (2015), in the case of wine lees hydrolysis. The effect of pH value on cheese whey hydrolysis yield was evaluated by conducting experiments within the range of 1 to 7, as it can be seen in Fig.4).

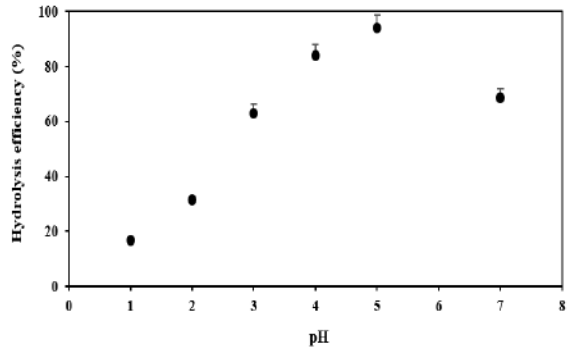

Fig.4: Effect of pH value on hydrolysis efficiency (percentage of total lactose concentration to glucose conversion) of cheese whey at optimum conditions (9 U mL^{-1} enzymatic activity, reaction time=12h; T=55°C and 1200 gL^{-1} initial cheese whey concentration)

Comparable lactose to glucose conversion yield were achieved at pH values in the range of 4.5 to 5, while hydrolysis yield was decreased at higher values. The pH value of 5M was determined as the optimum for cheese whey hydrolysis, using *A. oryzae* β-galactosidase, as an overall lactose to glucose yield of 93.98 % was achieved. To our knowledge there is no literature regarding unique cheese whey, delivered from Aegean islands, hydrolysis, although several researchers have studied hydrolyses of agroindustrial and industrial food processing by-product streams, using commercial or crude enzymes (Souza Moreira *et al*., 2012; Dimou *et al*.; 2015; 2016a; Lapena et al., 2018). So, in this study the highest total lactose hydrolysis of unique indigenous "Aegean" delivered mixture of Greek cheese whey, achieved at 55°C after 12h of enzymatic optimized process. Initial concentration of β-galactosidase preparation, highly affected final glucose production as well as lactose hydrolysis yields. Higher initial concentration of β-galactosidase preparation (for instance 12 gL^{-1}) did not further increased cheese whey hydrolysis efficiency. Dimou *et al*. (2015), studying enzymatic hydrolysis of wine lees, concluded that the initial enzymatic activity of crude *A. oryzae* enzymes highly affects hydrolysis yield. In this study, the maximum degree of hydrolysis achieved was approximately 94 %, higher than that observed by Haider *et al*. (2009), who studied whey lactose hydrolysis process, using β-galactosidase, of 0.88 UmL^{-1} and 0.44 UmL^{-1} concentrations, at 37°C, respectively. The authors reached a maximum degree of hydrolysis of 70%, at the end of enzymatic activity reaction (after 12h). The results obtained regarding unique cheese whey hydrolysis of Greek Aegean islands, are very satisfactory.

3.3 Development of industrially feasible process for the production of innovative glucose rich streams, suitable for novel food applications

The optimized enzymatic as well as acid catalyzed hydrolysis of unique Aegean delivered cheese whey was evaluated in a short industrially feasible 6 h reaction process, aiming at producing a glucose-rich hydrolysate that could find possible application, towards the production of "novel" functional foods with nutritional value, addressed to those who suffer from lactose intolerance. Glucose production, throughout acid as well as enzymatically catalyzed reaction, under optimum conditions in a 6-h process, is presented in Fig.5.

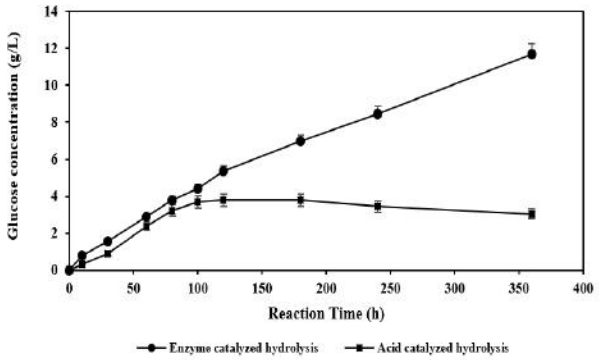

*Fig.5: Glucose concentration from cheese whey hydrolysis catalyzed either by acids[*1,*3] or β-galactosidase[*2,*3] after 6h of reaction, at optimum conditions for each process*

*[1]optimum conditions for acid hydrolysis of cheese whey: temperature=100 °C; pH=1; *[2]optimum conditions for enzymatic hydrolysis of cheese whey: temperature=55 °C; pH=5; initial enzymatic activity=9 U/mL *[3] The results are expressed as the mean values of three replicates (±SD)

After 6 h of cheese whey hydrolysis, hydrolysis yield is approximately 74 %, regarding enzymatically conducted process, while hydrolysis yield achieved through the acid catalyzed process is almost 18%. Thus, enzymatically derived hydrolysates could be possibly used for the production of novel lactose free products or low lactose products substituting other types of common commercial sweeteners. These results are very promising for further utilization of enzymatically catalyzed cheese whey in industrial food sector either substituting commercial nutrient sources such as glucose syrups and other saccharides. Lactose intolerance refers to syndromes such as diarrhea, abdominal pain, flatulence, and/or bloating that occur after ingestion of lactose containing food products. In fact an individual who suffer from lactose intolerance cannot digest or absorb lactose owning to a genetically programmed decrease in intestinal galactosidase (lactase) that occurs after weaning. This is a

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condition well known as resistance lactase pathophysiology or attributed to the damage caused to the lining of the epithelial cells in the digestive tract (Tomar *et al*., 2014). Lactose intolerance may be troublesome but in fact is not considered a condition that needs medical treatment, when individuals follow a diet that contains food products free or almost free of lactose. Thinking that 75% of the world's adult population is lactose or almost lactose intolerant the production of novel food free of lactose is of high significance. Besides, it has been reported that reduction of lactose concentrations from 70 to 80% in food products is enough for the vast majority of those who suffer from lactose intolerance (Szilagyi *et al*., 2015). So, enzymatic hydrolysis, if considered a treatment to cheese whey, prior to its industrial application for the designing and production of novel functional foods, could possibly improve the sensorial and technological properties of novel bio-produced functional products, addressed to those who suffer from lactose intolerance. Furthermore, it has been reported that *β*-galactosidase treatment of milk, could be possibly applied, as a bioprocessing stage, toward the production of sweeteners (Panesar *et al*., 2010).

3.4 Biocarotenoids and ergosterol production by *R. glutinis* cultivations, using cheese whey hydrolysates as nutrient supplements

Enzymatically derived cheese whey hydrolysates used as feedstocks, for the production of carotenoids and ergosterol, using *Rhodotorula glutinis* CCY 20-2-26.

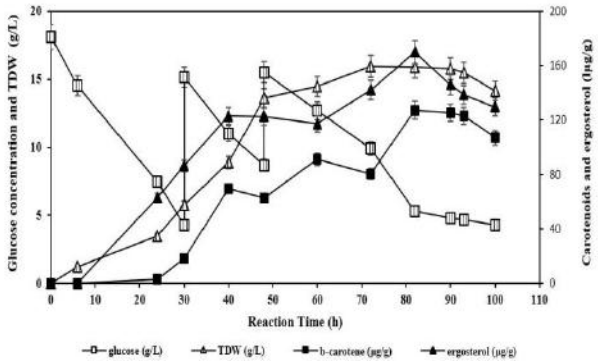

Fig. 6: Consumption of glucose as well as production of total dry weight (TDW*), carotenoids (b-carotene*) and ergosterol* using enzymatically derived cheese whey hydrolysate as nutrient supplements, during R glutinis fed batch fermentation*

* The results are expressed as the mean values of three replicates (±SD)

As it can be seen in Fig.6, *R. glutinis*, displayed, a two-phase growth character, characterized by a prolonged stationary phase. This could be probably attributed, to the ability of yeast cells to utilize supplementary energy resources such as lipids formed during yeast growth period. Furthermore, carotenoids as well as ergosterol production, during growth of *Rhodotorula glutinis* presented some fluctuations, with some local major and minor, as it can be seen in Fig.6. The major carotenoids produced by *R. glutinis* cells, under optimized conditions (T=26ºC; agitation speed=200-500rpm, pH=6.2-6.5) in bioreactor fed batch fermentations as well as preliminary shake flask cultivations (data not shown), using cheese whey hydrolysates as nutrient supplements was b-carotene (almost 0.95 g b-carotene/g total carotenoids). The maximum carotenoid production (carotenoids per dry weight) as well as ergosterol production (ergosterol per dry weight), using cheese whey hydrolysates (enzymatically derived), as bioprocessing feedstock achieved when the initial glucose concentration of enzymatically derived cheese whey hydrolysates was 18 gL^{-1} after 82 h of cultivation *R. glutinis* (while *R. glutinis* growth has already reached stationary phase) as it can be seen in Fig. 6. Ergosterol observed partly as the additional parameter of biomass quality, while monitoring competition of two specialized branches of isoprenoid pathway, which is used for the biosynthesis of both carotenoids and sterols (Britton *et al*., 1995). The production of ergosterol was very similar to the production of b-carotene (Fig.6), even though these metabolites are formed in competitive branches of isoprenoid metabolic pathway. More specifically, the highest production of 127.3 ± 0.41 $\mu g g^{-1}$ (or 2023.03 ± 0.41 $\mu g L^{-1}$), 170.78 ± 0.38 μg g-1 (or 2703.917 ± 4.37μg L^{-1}) and 15.89 ± 0.05 gL^{-1}, of total carotenoids, ergosterol and total dry weight, respectively achieved, using as feedstock cheese whey hydrolysates of 18 gL^{-1} glucose, enriched with 0.15 gL^{-1} $MgSO_4 \cdot 7H_2O$, 5 gL^{-1} K_2HPO_4, 5 gL_{-1} yeast extract and 5 % (wv^{-1}) NaCl, while pH was adjusted to the range of 6.2-6.5.

Under the same conditions, when deproteinized cheese whey prepared by acid hydrolysis process, used as fermentation feedstock, 59.54 ± 0.73 $\mu g g^{-1}$ (or 210.57 ± 1.12 $\mu g L^{-1}$) carotenoids, 53.49 ± 1.16 $\mu g g^{-1}$ (or 189.16 ±1.5 $\mu g L^{-1}$) ergosterol and 3.53 ± 0.05 gL^{-1} total dry weight produced, after 60h of fermentation. This low metabolite production during *R. glutinins* growth, using acid hydrolysates could be possibly attributed to inhibitory by-products produced during acid catalyzed cheese hydrolysis process (Baek *et al*. 2008). Preliminary experiments conducted, using crude cheese whey hydrolysates as nutrient supplements, led to approximately, under the same conditions led to 87 % less carotenoids and ergosterol production, highlighting the incapability of the studied strain to consume lactose. Taking into consideration all the above, is obvious that enzymatic derived cheese whey hydrolysates, form a better nutrient supplement for *R. glutinis* CCY 20-2-26 growth and concurrent metabolite production.

Very few studies have been published, using cheese whey as bioprocessing feedstock for concurrent carotenoid and ergosterol production by *Rhodotorula glutinis* strains. Kanzy *et al*. (2015), studying potential production of carotenoids using cheese whey as nutrient supplement reported that the maximum biomass (13.95 gl^{-1}) and volumetric carotenoid production (6.544 mgL^{-1}) were scored by an isolated *R. glutinis* strain after 120 h incubation at 30 °C and pH 6.6 in a medium containing 3% NaCl. Aksu *et al*. (2005) studied the production of carotenoids by *R.glutinis*, evaluating potential usage of low-cost substrates (glucose, sucrose from molasses and lactose from cheese whey) as fermentation media. The researchers concluded that the highest concentration of total carotenoids was obtained in a medium containing 20 gL^{-1} molasses sucrose. The highest specific carotenoid yield (35.5 mgg^{-1}) was reached when initial cheese whey lactose was equal to 13.2 gL^{-1}. To the best of our knowledge the results presented regarding concurrent production of carotenoids and ergosterol, are among the highest in literature cited publications using *R. glutinis* as microbial strain. Furthermore, it is of high significance to mention the genuity of this research regarding the revalorization of a unique by-product stream to value-added biomolecules with nutritional interest and several industrial food applications.

IV. CONCLUSION

In this study potential biocarotenoid production as well as other metabolite production such as ergosterol, using cheese whey derived from Aegean islands, as generic feedstock though the designing, development and optimization of a series of biochemical, chemical and biotechnological processes was evaluated and verified. Enzymatically derived cheese whey hydrolysate seems to be a better source for both bio-carotenoid and ergosterol production, compared to either untreated cheese whey or acid catalyzed cheese whey hydrolysates, under optimized conditions. Cheese whey hydrolysates produced via optimized enzymatic hydrolysis could possible find several applications for the production of carotenoids, ergosterol and other novel functional products such as novel lactose free food preparations or novel functional foods enriched with bio-carotenoids and/or bio-ergosterol. Integration of cheese whey, which is a low-cost substrate to nutritionally value-added products such as biocarotenoids, ergosterol and glucose rich streams free or semi-free of lactose is of great academic and industrial interest, while satisfying the principles of sustainable development and opening new areas in revalorizing by-product streams to value added products. These value-added products could find several applications in industrial food sector toward the production of novel enriched with bio-carotenoids and ergosterol products as well as innovative food products free or semi free of lactose, using enzymatically derived cheese whey as alternative source of sweetener.

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CONFLICTS OF INTEREST

The authors confirm that have no conflict of interest.

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