VOL. 12, NO. 10, OCTOBER 2017 ISSN 1990-6145
ARPN Journal of Agricultural and Biological Science

www.arpnjournals.com

PRELIMINARY INVESTIGATION OF REUSE OF RICOTTA-CHEESE WHEY *(scotta)* AS SUBSTRATE FOR THE GROWTH OF *Rhodotorula glutinis* INTENDED FOR THE PRODUCTION OF CAROTENOIDS

Josè Evangelista Ribeiro[1], Mina Martini[2, 3], Andrea Andreucci[4], Iolanda Altomonte[2], Carlo D'ascenzi[2], Margherita Marzoni[2], Simona Nardoni[2], Roberto Amerigo Papini[2], Stefania Perrucci[2], Federica Salari[5], Amanda Marilia Da Silva Sant'ana[1], Carlo Sorce[4] and Francesca Mancianti[2]

[1]Department of Food Engineering, Federal University of Paraíba, João Pessoa, Brazil
[2]Department of Veterinary Sciences, University of Pisa, VialedellePiagge, Pisa, Italy
[3]Interdepartmental Research Centre "Nutraceuticals and Food for Health", via delBorghetto, Pisa, Italy
[4]Department of Biology, University of Pisa, via Luca Ghini, Pisa, Italy -56126 Pisa, Italy
[5]Interdepartmental Centre of Agro-EnvironmentalResearch "Enrico Avanzi" University of Pisa, via Vecchia di Marina, S. Piero a Grado, Pisa, Italy
E-Mail: mina.martini@unipi.it

ABSTRACT

The ricotta-cheese whey, also called *scotta*, is the main by-product of ricotta-cheese manufacture process.This by-product is rich in nutrients that could make it a good substrate in biotechnological processes for the production of commercial high-value compounds. Nowadays, the demand for natural pigments and health-promoting food ingredients has increased. Carotenoids present a high commercial value as it is widely used in food, feed, pharmaceutical and cosmetic industries. A wide range of microorganisms and *Rhodotorula* genera yeast are able to produce carotenoids. The aim of this study was to evaluate the potential re -use of ricotta cheese whey (*scotta*) as substrate for the growth of *Rhodotorula glutinis* intended for the production of carotenoids. Under the cultivation conditions used, *R. glutinis*strain showed similar ability to grow and to produce carotenoids when cultivated in *scotta* compared to a semi-synthetic substrate. Under the cultivation conditions used, *R. glutinis* strain showed similar ability to grow and to produce carotenoids when cultivated in scotta compared to the semi-synthetic substrate. Thus, the results showed that *scotta* has potential to be used in a biotechnological process to obtain a high-value molecule. Further investigations are needed to test if changes in the cultivation parameters and the hydrolysis of lactose could improve the growth and the amount of cellular β-carotene.

Keywords: dairy by-products, *scotta*, carotenoids, yeast.

INTRODUCTION

Waste management is a crucial point for dairy plants for the high organic matter and high nutrient levels contained in dairy effluents.

Ricotta-cheese whey, also called *scotta*, is the main by-product of ricotta-cheese manufacture process. Ricotta-cheese is produced after the cheese making process, from raw residual cheese whey; fresh milk (up to 10%), milk fat and an acid solution of salts can also be added. The obtained mixture is maintained at high temperature (85-90 °C) to promote the precipitation of most of whey proteins that make ricotta-cheese. The liquid solution remaining after ricotta-cheese separation is called *scotta* and has different characteristics compared to cheese whey [1]. *Scotta* is widely produced in southern Europe and particularly in Italy where it represents a by-product to be disposed of [2]. The most of *scotta* is used as supplement feed for livestock. However, this by-product is rich in lactose and contains nitrogen, hydrosoluble vitamins and a variety of minerals that could make it a good substrate in biotechnological processes for the production of commercial high-value compounds.

Carotenoids are a group of over 600 molecules which fulfill diverse functions; dietary carotenoids are converted to vitamin A, are antioxidants and scavenger of oxygen radicals. Epidemiological evidence and experimental studies suggest that carotenoids enhance the immune response, inhibit the onset of many age-related diseases in which free radicals are thought to play a role in initiation, such as arteriosclerosis, cataracts, multiple sclerosis and cancer [3].Carotenoids and β-carotene are popularly used as natural colorants and antioxidants in food, feed, cosmetic and pharmaceutical products [4].

Scientists and consumers are interested in natural colorants due to the negative health effects of synthetic colorants [3] and because of the stringent rules and regulations applied to chemically synthesized/purified pigments.

The microbial production of carotenoids goes beyond the problems of seasonal and geographic variability of the extraction from vegetables and allows economic advantages if low-cost substrates are used. Various sources of carbon and nitrogen from agro-industrial origin (grape must, beet molasses, soybean flour extract, corn flour extract, cheese whey, fermented radish brine and sugar cane molasses) were already evaluated for cultivation of yeasts, with the purpose of developing a low-cost culture medium for carotenoid production [4,5,6].

Although several studies have proved the viability of using *scotta* as a substrate for the production of high-value products, such as bio-ethanol and lactic acid [1,2], to the best of our knowledge there are no reports about the use of *scotta* as a substrate to grow carotenoid-producing yeasts. Despite its large availability and low

price, this by-product is still poorly used in biotechnological processes.

Rhodotorula yeasts are non-photosynthetic organisms, widely distributed in nature, which can biosynthesize characteristic carotenoids [7,8]. *Rhodotorulaglutinis* is generally recognized in the literature as a good producer of carotenoids [9].

The aim of this study was to investigate the potentiality of reuse of *scotta* as substrate for the growth of *R. glutinis* intended for the production of carotenoids and compare the results with Malt Extract Broth (MEB), the first choice substrate for its cultivation.

MATERIALS AND METHODS

Microorganism

The yeast isolate was obtained from poultry droppings immediately after being shed and transported to the laboratory. The sample was dissolved in 2 mL of sterile water added with 50 µL mL^{-1} gentamicin and seeded onto Malt Extract Agar (MEA) supplemented with biphenyl 0.01%. The plates were incubated at 25 °C and daily examined over a 7 days period. To avoid contamination from fast-growing molds and/or bacteria, subcultures were achieved from day 4 post inoculation onto MEA until pure yeast colonies were obtained. *Rhodotorula* species isolates were screened macroscopically by evaluating texture and colonies color, and microscopically by culturing on cornmeal-Tween 80 agar.

The isolates were identified via the carbohydrate assimilation pattern, using the ID32C galleries (bioMérieux Italia SpA, Roma), along with a nitrate assimilation test. Definitive identification of *R. glutinis* was achieved by amplification and sequencing of ITS region of ribosomal DNA (rDNA). Genomic DNA was extracted from single colonies using the method described by Nunes*et al.*[6]. PCR and sequencing procedures were performed with the universal primers ITS1 and ITS4 [10]. Sequences were assembled and corrected by visual analysis of the electropherogram using Bioedit v.7.0.2, then compared with those available in GenBank using the basic local alignment search tool program [11] to assign the species.

Culture media

The *scotta* used in this study was supplied by dairy industries located in Toscana region, in Italy. The *scotta* was obtained from ricotta-cheese manufactured starting from a mixture of bovine and ovine cheese whey (90% and 10%, respectively). The physicochemical characterization was carried out for the following parameters: pH, acidity (g lactic acid 100 mL^{-1}), reducing sugars (g lactose 100 mL^{-1}), dry material, fat, ash content, total nitrogen, calcium, magnesium, phosphorous, sodium, potassium, zinc, iron and manganese. All the analysis was carried out according to the methodologies proposed by AOAC [12]. As a control, malt extract broth (MEB) was used as a semi-synthetic growth medium for the cultivation. The composition per liter was as follows: peptone (1 g), glucose (20 g) and malt extract (20 g).

Growth conditions

Yeasts cells at log phase were transferred to MEB (100%)with an inoculum concentration of 10^7 cells mL^{-1}. Yeasts were grown in Falcon tubes using 100% *scotta*; MEB was used as control. Tubes were exposed to visible light for 10 hours, maintained at room temperature and manually shaken until processed. The cultivation was carried out up to 28 days to evaluate the changes in the pigments accumulation and in the carotenoids profile along a large cultivation period. The long cultivation time was adopted due to the investigative character of the experiment. Each experiment was repeated at least three times with two replicates.

Analytical methods

Yeast biomass

The total biomass was quantified after 7, 14, 21 and 28 days of cultivation. A culture medium sample was collected and centrifuged at 6000 rpm for 5 min, the supernatant was discarded and the fresh biomass was weighted.

Carotenoids extraction and β-carotene quantification

Carotenoids extraction was carried out according to Cutzu *et al.*[13], modified as follows. Cultures were harvested by centrifugation at 6000 rpm for 5 minutes, then the supernatant was discarded and the pellets were frozen at -20 °C for 24 hours. The thawed cell pellet was re-suspended in 2 mL DMSO pre-heated at 60 °C, added with glass beads (0.5 g), vortexed for 2 min and incubated at 60 °C for 5 min. 2 mL acetone, 2 mL petroleum ether and 2 mL NaCl 20 % were sequentially added. Then the mixture was vortexed for a total of 5 min and centrifuged at 6000 rpm for 5 min. The upper petroleum ether layer containing the extracted carotenoids was transferred into a separatory funnel and washed five times with cold distilled water to remove all the DMSO from the petroleum ether phase.

The analysis of carotenoids was conducted using a SpectraSystem HPLC instrument equipped with a UV-VIS detector (Thermo, Rodano, Italy). The column was a Kinetex C18, 250 x 4.6 mm ID, 5 µm particle size (Phenomenex, Torrance, CA, USA), eluted at 1 ml min^{-1} with solvent A (acetonitrile: methanol: Tris buffer 0.1 M pH 8 84:2:14) and B (methanol:ethyl acetate 68:32), according to the following program: 100% solvent A for 4 min, then a linear gradient from 0 to 100% B in 10 min, followed by 100% B for 15 min. The β-carotene peak was identified by comparison of the retention time of sample peaks with that of a pure β-carotene standard (Extransynthese, Lyon, France). β-carotene quantification was carried out by reference to a standard curve.

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA). Average values were compared using Tukey's test. Statistical analyses were

performed using software *Statistica*®, version 7.0 (Statsoft, USA). Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSIONS

Physicochemical characterization of *scotta*

The physicochemical characteristics of *scotta* used as culture medium are shown in Table-1. As reported in the literature the main component present in the *scotta* is lactose [1]. This disaccharide is a suitable carbon source for many microorganisms [7]. The amount of nitrogen was similar to the results of Sansonetti *et al.*[1], in *scotta* produced from bovine raw cheese whey.

The variety and the concentration of minerals found in *scotta* support its application in biotechnological processes. In fact, El-Banna *et al.*[14] observed that supplementation of culture media with mineral salts resulted in an increase in cellular carotenoids from *R. glutinis var. glutinis*. These authors also suggested that the higher production of carotenoids was due to a stimulatory effect of cations on carotenoid-synthetizing enzymes. Moreover, metals such as potassium have important functions in microorganisms [15].

Table-1. Composition of the *scotta* utilized in this work.

Parameter	Mean ± standard deviation
pH	6.02 ± 0.03
Acidity (g lactic acid 100 mL^{-1})	0.155 ± 0.006
Reducing sugar (Lactose) (g 100 mL^{-1})	4.07 ± 0.21
Dry material (g 100 g^{-1})	7.75 ± 0.13
Total nitrogen (g 100 mL^{-1})	0.096 ± 0.017
Fat (g 100 g^{-1})	0.19 ± 0.00
Ash content (g 100 g^{-1})	0.94 ± 0.00
Ca (g 100 g^{-1})	0.048 ± 0.004
Mg (g 100 g^{-1})	0.030 ± 0.003
P (g 100 g^{-1})	0.093 ± 0.002
Na (g 100 g^{-1})	0.200 ± 0.004
K (g 100 g^{-1})	0.119 ± 0.009
Zn (mg 1000 g^{-1})	73.125 ± 0.884
Fe (mg 1000 g^{-1})	164.375 ± 0.884
Mn (mg 1000 g^{-1})	3.125 ± 0.884

Yeast biomass

The experiments showed that the growth of *R. glutinis* strain tested was satisfying when *scotta*was used as substrate. Regarding the total biomass (Figure-1), a significant difference ($p < 0.05$) between the two media was observed only at 21 days, when the MEB medium showed a slightly higher biomass concentration than *scotta*. The total biomass was similar for the rest of the cultivation period, showing that *scotta* can be a potential substrate to grow *R. glutinis* yeast, as the results were comparable to that obtained for the semi-synthetic medium. Concerning the composition of *scotta* (Table-1), we can conclude that this cheap substrate may support the growth similarly to MEB and may be used as the unique source of nutrients. Regarding the growth along the cultivation time, the amount of biomass for the yeast cultivated in *scotta* did not change significantly, whereas a decrease ($p < 0.05$) in the biomass was observed for the yeast cultivated in MEB after 21 days of cultivation.

It must be noted that the main carbon source found in *scotta* is lactose and that lactose-assimilating yeasts are rarely found in natural conditions [16]. Previous research has demonstrated the lack of ability to assimilate lactose by *R. glutinis*[17]. Furthermore, Aksu and Eren [7] observed that *R. glutinis* has a low tendency to grow in lactose containing media. It is tempting to speculate that a pre-treatment step to hydrolyze lactose could improve the growth through a higher consumption of this carbon source, as glucose and galactose can be well assimilated by this yeast [18].

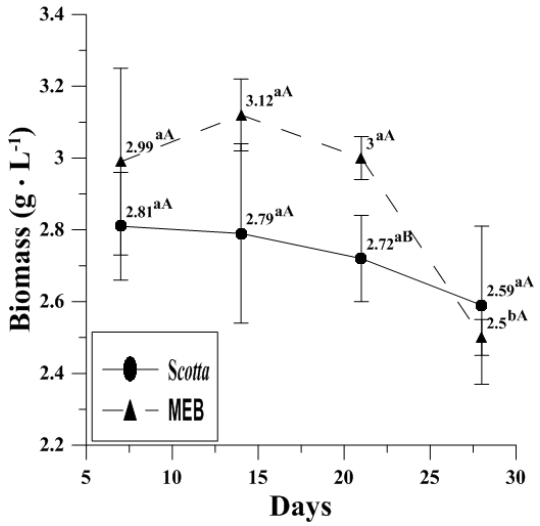

Figure-1. Biomass concentration along the cultivation time.

a,b: Different lowercase letters in the same plot mean significant difference along the cultivation time at 5% level of probability.

A,B: Different uppercase letters mean significant difference between the mediums for each cultivation time at 5% level of probability.

Data is expressed as mean of three replicates ± standard deviation.

Carotenoids production

The HPLC analysis enabled to identify β-carotene peak showing that the *R. glutinis* strain tested was able to produce carotenoids in the culturing conditions used. The amount of β-carotene ($\mu g \cdot g_{fw}^{-1}$) did not differ

between the growth media ($p > 0.05$) (Figure-2). It was previously reported that the cultivation time affects carotenoids production in the yeast cell [15]. In agreement with this observation, an increasing trend of β-carotene concentration during the cultivation time was observed in the present study, for the yeast cultivated in both *scotta* and MEB. Furthermore, the yeast grown in *scotta* showed a significant difference ($p < 0.05$) in the concentration of β-carotene between the first and third week, when the content of the pigment duplicated (Figure-2). In the yeast cultivated in MEB a higher β-carotene amount ($p < 0.05$) was observed after 21 days of cultivation, compared to the yeast cultured for 7 days. The *R. glutinis* biomass and β-carotene production in both substrates were lower in the present study than those by other researchers [1,4,19]. However, it is important to note that this study is a preliminary investigation, and through the optimization of culture conditions, such as the physical environment and the nutritional supply, it is possible to enhance cell growth and carotenoid biosynthesis, allowing the improvement of the process yield[20].

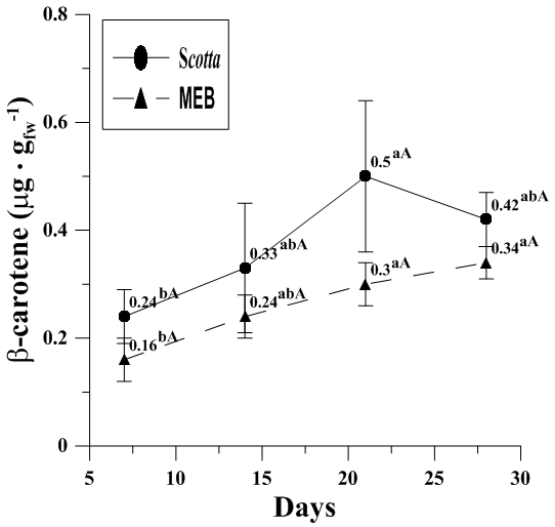

Figure-2. Cellular β-carotene content along the cultivation time.

a,b: Different lowercase letters in the same plot mean significant difference along the cultivation time at 5% level of probability.

A: The same uppercase letter means no significant difference between the mediums for each cultivation time at 5% level of probability.

Data is expressed as mean of three replicates ± standard deviation.

CONCLUSIONS

This study was an exploratory investigation on the use of a by-product of a dairy plant as a substrate for a *R. glutinis* strain intended to the production of carotenoids. Ricotta-cheese whey (*scotta*) was never tested before for this purpose. Under the cultivation conditions used, *R. glutinis* strain showed similar ability to grow and to produce carotenoids when cultivated in *scotta* compared to semi-synthetic substrate. Further investigations are needed to test if changes in the cultivation parameters and the hydrolysis of lactose could improve the growth and the amount of cellular β-carotene. The present research shows also a sustainability perspective as an agro-industrial by-product is exploited in a biotechnological process to produce a commercial high-value molecule.

ACKNOWLEDGEMENTS

Authors would like to thankPRA 2015- Progetti di Ricerca di Ateneo(University of Pisa Research Projects) and the Coordination for the Improvement of Higher Education Personnel (CAPES) for supporting this research.

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