

Algerian Yeast Strains: Isolation, Identification and Production of Single Cell Protein from Whey with Strain *Candida kefyri*

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Abstract—In this study, few samples than soft and baked cheese, yogurt, cow's milk, pasteurized milk and cheese whey were collected from dairy industries in the city of Boumerdes. The samples were cultured on yeast extract glucose chloramphenicol agar (YGCA) media. Twenty yeast strains were isolated from the culture. Ten of these strains were identified by their morphological and physiological properties. Betagalactosidase activity in the yeast strains showed that a strain *Candida kefyri* designated as LP1 had highest enzyme activity (up to 5000 EU/ml). To investigate the effectiveness of batch submerged fermentation of the yeast *C. kefyri* in cheese whey, we found that this strain have highest level of single cell protein (SCP), production (up to 19 g/l dry mass cell). The isolated yeast strain was examined for his ability in reduction of the chemical oxygen demand (COD). So, the results demonstrated a high level of reduction.

Index Terms—Beta-galactosidase, *Candida kefyri*, single cell protein, whey.

I. INTRODUCTION

The existence of pollution problems associated with agro-industrial wastes, costlier treatment options and increased need to save valuable resources have forced to encourage the utilization and bioconversion of waste into high value industrially useful products. Many by-products of the food industry are discarded in nature and thus constitute a source of pollution because of their quantity.

So, whey water, the greenish translucent liquid obtained after the precipitation of cheese is a very good source of lactose, proteins, vitamins and minerals. When, whey water is disposed to the environment without pretreatment, it pollutes the water bodies by increasing the biological oxygen demand in the range of 38,000 to 46,000 ppm as opposed to 200 ppm in case of sewage [1].

Approximately 1.50, 000 tonnes of Edam cheese and 2 million tonnes of whey are produced annually in Algeria. The recycling of whey is becoming valid and viable economic activity and is increasingly mentioned as a solution to some of the most serious problems of mankind. So, the huge amounts of whey considered as “waste” can potentially be used to produce various value added products like animal feeds, biofuels, enzymes, chemicals, etc. . . . However, the bioconversion of whey into single cell protein, (SCP), or ethanol has been performed in several countries [1], [2].

SCP could be produced from whey, with employing of

yeasts from different genera including *Kluyveromyces*, *Candida*, and *Trichosporon* capable of metabolizing lactose [3]-[6]. The most important characteristic of these single-celled organisms is their high protein content, ranging from about 40 to 80% of their dry weight on a crude protein basis.

In this study, some lactose fermentative yeasts strains were isolated and identified and the objectives of this study were to evaluate the capacity of the isolates to assimilate lactose, to investigate the effectiveness of batch submerged fermentation of the yeast in cheese whey for the production of SCP. Additionally; the COD removal was evaluated too.

II. MATERIALS AND METHODS

A. Sampling and Isolation of Yeast Strains

Several samples including soft and baked cheese, yogurt, cow's milk, pasteurized milk and cheese whey were collected from dairy producing factories in Algiers.

The samples collected in sterile 500 ml bottles and transferred to laboratory in a cooler box. The yeasts were enriched by inoculation of 10 ml (or 10 gr) of the sample in 90 ml of yeast extract glucose chloramphenicol agar (YGCA) medium, pH 4.5 containing 0.1 g/l chloramphenicol. The incubation was performed at 28 °C for 24 h with constant shaking of 120 rpm.

The yeast cells in media were examined using optical microscopy. The yeast strains were isolated on spread plates of Sabouraud chloramphenicol agar after making serial dilutions. The plates were incubated at 28 °C for 48-72 h. Colonies with distinct morphological differences were selected and purified by streaking on potato-dextrose agar (PDA) [7].

B. Measurement of Beta-Galactosidase Activity

According to Gunther and Burger [8], O-Nitrophenol β -D-Galactopyranoside (ONPG) assay was applied for calculation of beta-galactosidase enzyme activity in the yeast strains as previously reported [8]. Briefly; the isolated strains were inoculated in YPD medium and incubated at 28 °C for 12-18 h. One ml of yeast cultures (approximately 10^8 cells) at OD₆₀₀ was span down. Then, the cells were washed in 1ml cold Z buffer (0.06 M Na₂HPO₄, 0.04M NaH₂PO₄, 0.01M KCl and 0.001M MgSO₄). The activity of the beta-galactosidase enzyme was measured in the presence of ONPG at OD₄₂₀ nm and the Miller unit was calculated as follows:

Units=1000 × OD₄₂₀ / volume (1 ml) × time (min) × OD₆₀₀ (1)

C. Identification of the Yeast Strains

To identify the yeast strains capable of lactose fermentation, the isolated yeasts were cultured in Durham tubes containing 2% (w/v) lactose [9]. Then, the positive yeast strains for lactose fermentation were identified using the standard taxonomic key outlined according to Barnett *et al.* [10] and Yarrow [11]. Experimental results of physiological tests were analyzed by the software package of Barnett *et al.* [12]. Growth assays were repeated 3 times in 3 replicates.

D. Cultivation of Selected Strain on Whey Medium

Growth experiments were conducted in 5 L stirred tank bioreactor with a working volume of 3.8 L was employed in this experiment. 3 L ml of sterilized cheese whey medium was transferred to the bioreactor which was then inoculated with 300 mL of microbial yeast suspension. The bioreactor was maintained in conditions: 28 °C, pH 4.5 (by addition of 1 M HCl and 1 M NaOH throughout the fermentation), aeration 1 vvm, and 400 rpm. After inoculation the bioreactor was set to work for 22 h and samples were drawn every 2 h. To eliminate whey's proteins, it was clarified by heating at 100 °C for 15 min after adjusting the pH to 4.6 (isoelectric pH). After settling of the whey's proteins, the supernatant was ultra filtered and the greenish yellow liquid was sterilized at 115 °C for 10 min. We also supplemented the deproteinized whey with 0.5% (w/v) ammonium sulfate as the nitrogen [13].

E. Analytical

The cell dry weight was determined as previous work [14], [15]. Aliquot of the culture media was centrifuged at 5000 rpm for 15 min, after washing the biomass with Ringer serum; it was precipitated for a second time before measuring dry weight, followed by drying of the cells at 105 ± 1 °C for 2-3h.

In addition, the optical density of the culture was measured at 620 nm in a spectrophotometer. The lactose concentration was determined using sugar analyzer (YSI Model 27). Chemical oxygen demand was determined according to the procedures described in the Standard Methods for the Examination of Water and Wastewater [16]. The concentrations of ethanol in the samples were determined by high-performance liquid chromatography (HPLC) (Shimadzu Corp) using a Rezex ROA column (Phenomenex) at 63 °C and 4 mM H₂SO₄ as eluent at flow rate of 0.6 ml/min, equipped with a refractive index detector. Samples were pH-adjusted to 2.0–2.3 and filtered through a 0.45 µm membrane prior to injection into the vials.

III. RESULTS AND DISCUSSION

A. Phenotypic Characterization of Isolates

Pure cultures of 20 morphologically distinct yeast isolates dairy producing factories in Algiers were selected on the basis of the colony morphology. This study revealed that 10

out of 20 isolates were capable of growing on lactose. The characterization of these yeasts was based on conventional methods established by Barnett *et al.* [10], Yarrow [11] and Barnett *et al.* [12]. These methods rely on the definition of various morphologic and physiological characteristics of yeasts. Rapid methods currently marketed (API 50 CH) may validly, in some cases, replace the conventional approach of identification, for example yeasts encountered in hospitals. However, they are proving inadequate for dairy and food products [17].

Morphological criteria include the appearance of the cell, the aptitude of filamentation and sporulation, and the appearance of the liquid and solid media. Physiological criteria include the assimilation of a nitrogen compound, the assimilation and fermentation of different carbon sources, and the resistance to certain unfavorable environmental conditions (presence of antibiotic or high sugar concentration). According to Barnett *et al.* [10] data, 439 species of yeasts belonging to 57 different genera, were counted and are identified by these methods. Moreover, in the simplified dichotomous keys of the classification of yeasts, it is established that most of the yeasts present two modes of asexual divisions [18].

So, the first group included the genus *Schizosaccharomyces* which is divide by scissiparity, and the second group is composed of most of the other yeasts that breed vegetatively by budding. Among the isolated yeasts, the asexual reproduction of the strains VLV, FR1 and FR4, respectively obtained from cow's milk, hard cheese and soft cheese, is characterized by the scissiparity.

However, according to Lodder [18] and most authors, only the *Schizosaccharomycoideae* are characterized by dividing by binary fission. According to the same author, this family includes a single genus. It is the *Schizosaccharomyces* genus, and is represented by some species like *S Japonicus*, *S pombe*, *Smalidevorans* and *Soctosporus*.

However, the isolated Strains FR1, FR4 and VLV have broadly the same morphological and sexual characteristics of the *Schizosaccharomyces* genre. Indeed, Lodder [18] and several authors characterize this genre by whitish cream colored colonies on a solid medium, the cells' shape is elongated, ovoid and spherical, and they shred to give a true mycelium that fragments into arthrospores.

Sexual reproduction is present, the spores are of a globular form - their number varies from 4 to 8 spores depending on the species. Nitrates are never assimilated by the genus *Schizosaccharomyces*. The assimilation and fermentation of various sugar compounds vary from one species to another. Comparing our results with those reported in data [10], [11], [12], [18], we can see that the yeast strains FR1, FR4 have the same characteristics as the strain *Schizosaccharomycesoctosporus*, this latter species was isolated by Lodder [18], from sugar cane molasses. It is well spread in sweet food, but rarely in dairy products.

The presence of *Schizosaccharomycesoctosporus* in cheeses used as sample isolation is possibly due to his unexpected presence in the milk powder, the latter is a staple

ingredient of recombined milk used for the cheese formulations in the Algerian dairies.

However, the yeast strain VLV showed the same characteristics as the species *Schizosaccharomyces malidevorans*. The presence of this yeast in a dairy product such as cow's milk has not been previously demonstrated. The natural biotype of this strain is not known [18]. This is yeast that has been isolated for the first time from grapes [19]. It is characterized by its inability to assimilate maltose, but easily grows on glucose.

Thus, among the ten yeast strains isolated, the strains FR4, FR1 and VLV belong to the genus *Schizosaccharomyces* and respectively to the *S.octosporus* and *S.maldevorans* species.

Furthermore, Graham [19] claims that when yeast is very infrequently isolated, it can be assumed that it is rare in nature or possibly undefined. In addition, it should be noted that most of the yeasts generally have a particular geographical location.

Finally, among the 10 strains of yeasts isolated, there are three yeast species to adopt a scissiparity mode of division, among the remaining strains none of them are divided by bipolar budding and can therefore not be one the following genera: *Kloeckera*, *Wickerhamii*, *Nadsonia* and *Hanseniaspora* cited by Hupert [20].

Besides, all our isolated strains breed vegetatively by multipolar budding. Therefore, the yeast genera mentioned above are to be excluded.

According to Lodder [20] and Barnett *et al.* [21] data, the criterion sporulation is the basis of the dichotomy between *Ascosporogenous* yeasts (Fungi imperfecti) when physiological tests do not allow, by themselves, to distinguish these large types of yeast. Indeed, some anascosporogenous yeasts exhibit identical characteristics to those of ascosporogenous species.

Thus, the study of the sporulation ability allows distinguishing between the isolated strains of yeasts VL1, VY1, FR2, FR3, and VKL1 and the strains LP1 and VY2; the latter two strains have no ability to form ascospores or other form of sporulation. Thus, the strains VY2 and LP1 have similar characteristics with the *Candida* genera, *Cryptococcus* and *Torulopsis* described by Lodder [18] and other authors. In order to distinguish between both the genera *Cryptococcus* from *Torulopsis*, the same author propose to include in the genus *Cryptococcus* all species that can assimilate inositol as the single carbon source. In addition, he also adopted the assimilation of inositol test and has considered it as an important criterion in distinguishing between the both genera *Cryptococcus* and *Torulopsis* [18].

In fact, *Cryptococcus* genus assimilates only inositol. So, *Torulopsis* species are unable to assimilate this carbon compound. Yet, comparing our results (Table I), we note that the strains LP1 and VY2 assimilate inositol and other carbon sources.

About the both genera *Candida* and *Torulopsis*, it is often difficult to distinguish between them because they have much in common. That is why some authors adopted the filamentation test or the formation of pseudomycelium as a

distinctive criterion. Indeed, most *Candida* species are characterized by their ability to form a well-developed pseudo mycelium, while, it is not present or rudimentary in the genus *Torulopsis*.

However, yeast strains LP1 and VY2 form an abundant pseudomycelium. Given this range of distinctive criteria, we could identify the strains LP1 and VY2 and join them to *Candida* genus. According Lodder [18] in the genus *Candida* the cells are globular, ovoid, cylindrical or elongated but never ogival and dived by budding and the filament cells give an abundant pseudomycelium. Thus defined by these authors, *C. kefyri* and *C. pseudotropicalis* have the same characteristics as the isolated yeast strains LP1 and VY2.

TABLE I: IDENTIFICATION OF YEASTS ISOLATED FROM DAIRY PRODUCER IN THE CITY OF ALGIERS

Isolate strain	VLV	FR4	FR1	LP1	VY2	VL1	FR3	FR2	VKL1	VY1	
	<i>glucose</i>	+	+	+	+	+	+	+	+	+	
<i>galactose</i>	+	+	+	+	+	+	+	-	-	-	
<i>saccharose</i>	+	+	+	+	+	+	+	-	-	-	
<i>melibiose</i>	-	-	-	-	-	-	-	-	-	-	
<i>maltose</i>	-	-	-	-	-	-	-	+	+	-	
<i>lactose</i>	+	+	+	+	+	+	+	-	-	-	
<i>trehalose</i>	-	-	-	-	-	-	-	-	-	-	
<i>cellobiose</i>	-	-	-	-	-	-	-	-	-	-	
<i>glucose</i>	+	+	+	+	+	+	+	+	+	+	
<i>galactose</i>	+	+	+	+	+	-	-	-	-	-	
<i>saccharose</i>	+	+	+	+	+	+	+	+	+	+	ASSIMILATION
<i>melibiose</i>	-	-	-	-	-	-	-	-	-	-	
<i>maltose</i>	-	+	+	+	-	-	-	+	+	-	
<i>lactose</i>	+	+	+	+	+	+	+	-	-	-	
<i>trehalose</i>	-	+	+	+	-	-	-	-	-	-	
<i>arabinose</i>	-	-	-	-	-	-	-	-	-	-	
<i>xylose</i>	+	+	-	-	+	-	-	-	-	-	
<i>cellobiose</i>	-	-	-	+	+	-	-	-	-	-	
<i>nitrate</i>	-	-	-	-	-	-	-	-	-	-	
<i>inositol</i>	n d	n d	n d	+	+	n d	n d	n d	n d	n d	
Species	<i>Schizosaccharomyces</i> sp.	<i>Schizosaccharomyces</i> sp.	<i>Schizosaccharomyces</i> sp.	<i>Candida kefyri</i>	<i>Candida kefyri</i>	<i>Kluyveromyces fragilis</i>	<i>Kluyveromyces Lactis</i>	<i>Kluyveromyces Lactis</i>	<i>Kluyveromyces Lactis</i>	<i>Kluyveromyces marxianus</i>	

The distinction between the two first, lies in their ability to assimilate xylose, a sugar easily used by *C.pseudotropicalis*.

According to the results, the LP1 and VY2 strains, respectively isolated from pasteurized milk and yoghurt are linked to the *Candidakefyr* species. Wijeyaratne [5] reported in his works an abundant presence of this yeast in dairy products such as milk and cheese, it is part of their normal microbial flora, considering its ability to use lactose, dominant sugar in milk.

In their study, Mlimbila *et al.* [22] demonstrates that diverse yeast species existed in the examined Tanzanian traditional (*mtindi*) and industrially fermented milk (yoghurt) in which *mtindi* from Mwanza contains the most diverse yeast flora using ribosomal DNA-Polymerase Chain Reaction (PCR) amplification and sequencing of the domains D1/D2 of the 26S rRNA gene. The identified yeasts were *Candida tropicalis*, *C. pararugosa*, *Candida* sp. and *Pichiasp*.

Regarding VL1, VY1, FR2, FR3 and VKL1 strains, in addition to their asexual multiplication which occurs by multipolar budding, they have the ability to sporulate and form ascospores, thus, the sporulation allows to exclude the anascosporogenic yeasts and to distinguish them from the previously described species.

According to some authors, such as Lodder [18], *Pichia* and *Kluyveromyces* genera have many properties in common with the isolated yeast strains VL1, S the ascospores are spherical, ovoid and their number varies from 1 to 16 ascospores per ascus. Nitrates are not assimilated by this genus. The membership of each of the isolated strains (VL1, VY1, FR2, FR3 and VKL1) to the corresponding species, like *kluyveromyces* genera, lies in the comparison of the made physiological tests, with those of the *kluyveromycesfragilis*, *kluyveromyceslactis* and *kluyveromycesmarxianus* species [18].

Thesesame species have very similar physiological characteristics. They differ from one another by the following features. The species *K. lactis* easily assimilates trehalose unlike *K. fragilis* and *K. marxianus* which are unable to assimilate sugar. However, according to our results (Table I), the strains FR2, FR3 and VKL1 assimilate well trehalose, therefore, they belong to the species of *K. lactis*. Furthermore, the difference between *K. marxianus* and *K. fragilis* lies in their speed to ferment lactose; indeed, *K. marxianus* species slowly ferment lactose while it is rapidly fermented by *K. fragilis* [18], [23], [24]. The achievement of this test, allowed us to identify the isolated yeast strains VL1 and VY1 and affiliating them respectively to the *K. fragilis* and *K. marxianus* genera.

Nevertheless, according to previous authors, the fungus flora of cheese and some dairy products consists of a yeast population in which the species of the genus *Candida* and *Kluyveromyces* are strongly represented. Dairy products are considered as their natural biotopes. To conclude with, we could isolateten different strains of yeasts from used dairy products. These yeasts strains are considered as lactic yeasts. The greater part of them is cultured from whey for the

production of single-cell proteins. Yeasts of the genus *Schizosaccharomyce* sare never used for this purpose, in fact, in stirred liquid culture, these yeasts develop hyphal forms once the medium is depleted of its nutrients.

B. Screening and Kinetic Analysis of SCP Production

The lactose metabolism by the yeasts was studied by several authors [23], [24]. In this study, 20 yeasts isolates were examined for lactose fermentation ability. Among them, 7 strains (LP1, VY2, VL1, FR3, FR2, VKL1 and VY1) were found capable of lactose fermentation. Enzyme activity of beta-galactosidase in yeast strains was measured. Among 07 yeast strains, the LP1strain (*Candida kefyr*) was found to have the highest enzyme activity, 5366 unit/ml (Table II). Also the VL1 strain (*Kluyveromyces fragilis*) and VKL1 strain (*Kluyveromyces lactis*) were showed high enzyme activity, 5182 and 5020 unit/ml, respectively.

Screening of lactose-fermenting yeast strains has been conducted by Ianieva *et al.* [23], among 162 strains isolated from various plants and 28 strains isolated from cheese, four yeast strains fermented lactose and were identified as *Candida kefyr*. The specific β -galactosidase activity of the studied strains was 1501–2113 IU/g dry biomass. The same authors found that the ability of strains *C. kefyr* C24 and C30 to produce ethanol from lactose was significantly inhibited by the increase in substrate concentration (100 g/L) [23].

TABLE II: BETA-GALACTOSIDASE ACTIVITY IN THE ISOLATED YEAST STRAINS

Isolate	Sample	Species	Beta-galactosidase activity (EU-ml) Using whey medium
LP1	Whey	<i>Candida kefyr</i>	5366
VY2	Cow Milk	<i>Candida kefyr</i>	4832
VL1	Yogurt	<i>Kluyveromyces fragilis</i>	5182
FR3	Dough Baked Cheese	<i>Kluyveromyces lactis</i>	4120
FR2	Dough Baked Cheese	<i>Kluyveromyces lactis</i>	4009
VKL1	Dough Baked Cheese	<i>Kluyveromyces lactis</i>	5020
VY1	Yogurt	<i>Kluyveromyces marxianus</i>	4646

In this study, the results showed that there was a direct relationship between lactase activity and biomass production in cheese whey. Batch fermentation of whey with *C. kefyr* was performed, at 28 °C, pH 4.5 (Fig. 1) with pH control. Fig. 1 illustrates the SCP production, lactose utilization, ethanol production and chemical oxygen demand. The low pH was

chosen to overcome bacterial contamination.

Lactose utilization started within 8 h, and all lactose was utilized after 16 h. Lag phase in SCP production was observed in this experiment, it lasted 8 hours of time. During the exponential growth phase, the biomass concentration increased exponentially with cultivation time and reached 19 g/l and lactose concentration depleted rapidly, however, the stationary growth phase reaching after 14 h and the lactose was reduced about 87.11% after 14 h.

In this study, the ultra filtration of the spent culture reduced in an effluent having total COD reductions of 94%. The biomass yield obtained in this case was greater than those reported *Torulopsis cremoris* and *K. fragilis* and *C. kefyfyr* and *C. valida* [25]-[27].

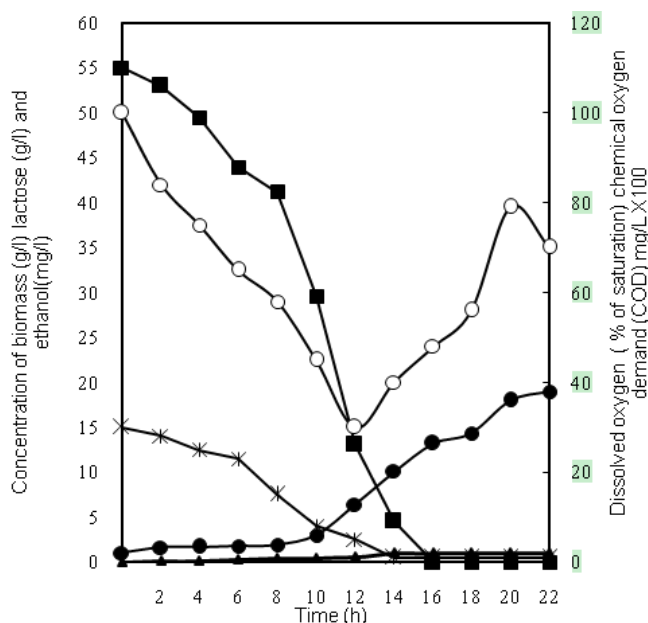


Fig. 1. SCP production during the growth of *C. kefyfyr* on cheese whey: concentration of biomass yield (—●—), lactose consumption (—■—), ethanol production (—○—), dissolved oxygen (—○—) and chemical oxygen demand (COD) reduction (—*—).

Increasing demand for protein sources of high nutrition value has stimulated the application of SCP in animal feed. With regards to these results we consider these yeast strains especially to be viable candidates for production of single cell protein from cheese whey. However, the reduction of the COD was studied by some authors such as Yadav *et al.* [28]. In this study, cell growth reached the end of the exponential phase after 22 hours and within this period most cells achieved COD reduction in Whey medium by 94% (Fig. 1), the final COD values ranged between from 150 to 100 mg/L in this batch culture.

IV. CONCLUSION

To promote high tonnages whey production by fermentation of single-cell protein is one of the most attractive ways. Production of *Candida kefyfyr* yeasts for food or feed is a bioprocess already exploited industrially. To be economically competitive, the production of yeast on whey must be performed with a maximum conversion of lactose

into biomass with high productivity in a fermentor. Together, the data showed that the isolated yeast strain LP1 could be of valuable application in bioconversion of cheese whey.

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