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Biological treatment of the sweet and acid whey by *Candida kefyr*

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ABSTRACT

The dairy industry produce large quantities of whey which is a byproduct causing a pollution once rejected in the environment without treatment. Therefore this study aims to treat the mixture of acid and sweet whey by coupling two processes. The primary physicochemical treatment of sweet and acid whey diluted to ¼, realized by coagulation-flocculation with initial pH 10 using 800 mg L^{-1} of the aluminum sulfate in combination with 400 mg L^{-1} of the polymer Zetag 48, has reduced the turbidity with 90%, orthophosphate with 98%, total nitrogen with 77%, COD with 17% and BOD5 with 11%. This treatment was effective in reducing turbidity, orthophosphate and total nitrogen which reached the discharge standards. The secondary biological treatment was realized by adding only Candida kefyr in suspension or in combination with Aspergillus niger or Saccharomyces cereviseae during 5 days. The best treatment was obtained using Candida kefyr enriched by 1g L^{-1} of ammonium sulfate as a source of nitrogen giving a reduction of 66% of the COD and 94% of the BOD5. Orthophosphate indicates a reduction of 95%. Total nitrogen was consumed by Candida kefyr, despite the addition of ammonium sulfate, and notes a final value of 47.3 mg L^{-1}.

Keywords: Sweet and acid whey, physico-chemical treatment, biological treatment, *Candida kefyr*, *Aspergillus niger*.

INTRODUCTION

The sweet whey issued from the fabrication of cooked or uncooked cheese, present at least 85% of the milk transformed into cheese (8 to 10 kg of milk are needed to make 1kg of Halloum or Akkawi cheese) [1]. As a major source of pollution in the dairy effluent, the whey is characterized by an extremely high load of organic matter, mainly lactose, which destroys aquatic life and causes pollution by asphyxiation of the receiving environment [2]. When the sweet whey and other waters are mixed, this mixture is diluted and organic matter content decreases, so the dilution is a preliminary step applied in all dairy industries discharging dairy effluent. For wastewaters, the COD/BOD5 ratio is less than 2 making them readily biodegradable. One liter of processed milk is about 60 g COD, which 50 g come from whey and only 10 g from whitewater [3].

In many countries, the cost of treatment systems can be a barrier to reach the discharge standards. In Lebanon, the whey is rejected by most industries directly in the wastewater. Thus, it's important to find a

simple, effective, economical and applicable technique on industrial scale to reduce environmental impacts generated by the dairy industry. A study realized by Ayeche and Balaska in 2010 [4], shows good results using lime which reduces 92% of materials in suspension and 83% of total phosphorous. This treatment was insufficient concerning the elimination of organic pollution. Results obtained by Kushwaha and al. in 2010 [5] indicate that optimum doses of 300, 800 and 500 mg L^{-1} of PAC, $FeSO_4$ and $(KAl(SO_4)_2.12H2O)$ respectively give a COD reduction of 69.2, 66.5 and 63.8 % respectively at pH 8. To ensure complete treatment, coagulation-sedimentation must be accompanied by a biological treatment to reduce the pollutant load and meet the standards. A study shows that biological treatment of dairy effluents by the bacterial strain *Alcaligenes sp.* MMRR7 allows obtaining a maximum reduction of COD of 62% during 5 days of incubation. But this bacterium is pathogenic and can contaminate the industry [6]. Baroudi and al. in 2012 [7] found that treatment by *Pseudomonas fluorescens* reduces the COD of 57.35% for 20 days and 75.49% for 40 days. In the other hand, treatment with *Bacillus pumilus* decreases the COD of 80.14% in 40 days which is considerate a long time for the dairy industry. A study centered on the production of lactic yeast on whey by continuous and batch fermentation shows that among selected and isolated strains from dairy products (*Candida kefyr, Kluyveromyces fragilis, Kluyveromyces lactis*), the results indicate a very good growth of *Kluyveromyces fragilis* and reduction of 99% of the COD during 24 h of incubation [8]. *Candida kefyr* (synonymous of *Candida pseudotropicalis*) was used in some studies as the most effective microorganism from yeasts which ferment lactose, for the conversion of lactose into ethanol through their ß –Galactosidase enzyme (lactase) [9,10]. This yeast is facultative aero-anaerobic isolated from dairy products and found in animals (mice). It is characterized by a rapid growth of 24 h, white to yellow creamy colonies. *Aspergillus niger* is a filamentous fungi, colonize in 24 to 48 h acid environments in aerobic conditions [11]. It is cosmopolitan, can be isolated from air or soil environment and frequently found in grains, fruits and moldy vegetables, dairy products, peanuts, used in the food industries to produce various acids such as citric acid [12] and also for the production of ß-galactosidase enzyme [13]. Due to the effectiveness of fungal biomass to degrade organic pollution of whey during a short time, this study seeks to provide a physic-chemical treatment of sweet and acid whey realized by coagulation-flocculation-sedimentation and optimized using a coagulant agent, aluminum sulfate, and a synthetic polymer Zetag 48, as flocculating agent. This treatment is then coupled to a biological batch treatment by adding only *Candida kefyr* in suspension or in a mixture with *Aspergillus niger*, enriched or not by a nitrogen source. The efficiency of this treatment was studied by the analysis and the monitoring of biomass and the chemical oxygen demand as measure indicators of the organic matter degradation.

Whatever the exploitation is, the direct discharge of dairy effluent into the environment is prohibited (Decree of 12 June 1996). Discharge standards for dairy effluents (acid and sweet whey + dairy whitewater) are determined by 35 mg L^{-1} Total suspended solid, 40 mg L^{-1} BOD5, 125 mg/l COD, 30 mg L^{-1} for total nitrogen (expressed as N), 10 mg L^{-1} for total phosphorus and pH between 6 and 9 [14].

MATERIALS AND METHODS

Sampling: Sweet and acid whey were collected from the industry Rene Moawad Foundation, a non-governmental organization (NGO), located in the North Country (Zgharta). Every sample is about 4 L, freshly collected after draining step, and stored maximum for a week in a refrigerator at 4°C.

Characterization of sweet whey: Physic-chemical analyzes are performed according to the methods described by AFNOR and Quebec to investigate the sources of pollution and characterize the rejected effluent. In order to evaluate the effectiveness of the treatment and ensure mitigation standards, measurements are performed on the sample which is a mixture of sweet and acid whey diluted to ¼ with water to approximate conditions to industrial scale where the industry reject the whey (generated with a volume 0.75 l L^{-1} of milk) mixed with whitewater (3 to 4 l L^{-1} of milk) en because dilution helps reducing pollution (cheaper and economic for the physic-chemical treatment). For the biological treatment, dilution is used to avoid the phenomenon of substrate inhibition due to a high concentration of carbon. The following parameters were studied: temperature, pH, turbidity, Chemical Oxygen Demand (COD),

Biological Oxygen Demand for 5 days of incubation (BOD5), Total Nitrogen (TN), and orthophosphate ($P\text{-}PO_4^{3-}$).

Coagulation-flocculation Assays: The various tests of coagulation-flocculation-sedimentation were conducted on dairy effluent on laboratory temperature (24 ± 2 °C) using a Jar-test, Stuart. The amount of coagulant, flocculent and the pH were optimized. In a series of 6 beakers, 250 ml of acid and sweet whey were mixed, diluted to ¼ and introduced, and then the coagulant agent, the aluminum sulfate, and the flocculent agent which is a solid synthetic polymer named Zetag 48, added with increasing doses during an agitation for a short time on a fast stirring (250 rpm 5 min^{-1}) followed by a slow stirring (40 rpm 2 min^{-1}). After 5 min of sedimentation, the supernatant was siphoned off to be analyzed.

Three optimization tests were performed:

- Fixation of the polymer dose with increasing doses of aluminum sulfate ($Al_2(SO_4)_3.18H_2O$): 400 to 2400 mg L^{-1}
- Fixation of the aluminum sulfate dose with increasing doses of the polymer: 400 to 2400 mg L^{-1}
- Variation of the pH: from 5 to 12 (adjusted by HCl or NaOH) in case of combination of optimal doses of aluminum sulfate and polymer.

During these three trials, turbidity is chosen as the indicator of the best treatment to determine the optimum pH, coagulant and flocculent dose. Other parameters were also measured at the end of the optimized physic-chemical treatment to evaluate its influence on the reduction of COD, BOD5, total nitrogen and orthophosphate.

Biological treatment Assays

Microorganisms: Strains of *Candida kefyr* ATCC 2512 and *Aspergillus niger* were taken from the Laboratory of microbiology on Azm Center for the Research on Biotechnology and its Applications. *Aspergillus niger* was isolated from the environment, while *Saccharomyces cereviseae* was simply isolated from the commercialized yeast. Strains were grown on Sabouraud dextrose agar (SM046, India) adjusted on the pH 5, at 37º C from 24 to 48h then left on bench.

First trial of the biological treatment*:* Three treatments were carried out in parallel by adding respectively *Candida kefyr*, a mixed culture of *Candida kefyr* and *Aspergillus niger* and a mixed culture of *Candida kefyr* and *Saccharomyces cereviseae* on primary treated whey by coagulation-flocculation, readjusted at pH 5-6 and saturated by oxygen under laboratory conditions. The cultures were incubated at room temperature on a shaker at 110 rpm min^{-1} during 5 days. In the end of this period, COD is measured in order to evaluate the efficiency of every treatment.

Second trial of the biological treatment*:* According to the first trial, *Candida kefyr* and *Aspergillus niger* are selected as the effective strains for the biological treatment of whey. Subsequently, four treatments of whey are performed in parallel and the control one is carried out to study the behavior of endogenous bacteria. The samples tested are:

- Coagulated-flocculated whey inoculated with *Candida kefyr*.
- Coagulated-flocculated whey inoculated by *Candida kefyr* enriched with 1g L^{-1} of a nitrogen source: ammonium sulfate $(NH_4)_2\ SO_4$.
- Coagulated-flocculated whey inoculated by *Candida kefyr* and *Aspergillus niger.*
- Coagulated-flocculated whey inoculated by *Candida kefyr* and *Aspergillus niger* enriched with 1g L^{-1} of a nitrogen source: ammonium sulfate $(NH_4)_2\ SO_4$.

This treatment is performed by fermentation in batch cultures at room temperature, at pH 5-6 and stirred in a shaker during 5 days with 5 min daily oxygenation. Mycelium growth is indicated during the treatment by a simple and rapid measure of the absorbance at 660 nm every day, the evolution of the organic matter is monitored by the measurement of the COD and several parameters are measured for a final characterization of the effluent. Before testing, every sample was centrifuged for 10 min at 110 rpm min^{-1} and the supernatant is tested.

RESULTS AND DISCUSSION

Whey characterization: The sweet and acid whey mixture is characterized by a high turbidity of 694 FTU which is due to the high load of colloidal particles and cheese particles in the whey. COD noted an average value of 68000 mg L^{-1} which reflects the amount of organic material in carbonaceous (lactose), nitrogen and phosphorous form. This value includes the particular and soluble COD. Biodegradable organic materials indicate a value of 34840 mg L^{-1} BOD5 and the COD/BOD5 ratio is equal to 1.95<2 where the effluent is biodegradable. The orthophosphate (inorganic P) is associated to colloidal particles of milk: casein [15] and equal to 112.4 mg L^{-1}. Total nitrogen found essentially as organic form, noted a value of 389 mg L^{-1} which mostly come from insoluble milk proteins such as casein and other soluble proteins (Table 1).

Table 1: Whey characterization

Analyzed parameters	Average values
pH	4.8
Temperature (^oC)	25
Turbidity (FTU)	694
COD (mg L^{-1})	68000
BOD_5 (mg L^{-1})	34840
Orthophosphate (mg P L^{-1})	112.4
Total nitrogen (mg L^{-1})	389

Coagulation-floculation Assays

Optimization of the dose of Aluminum sulfate

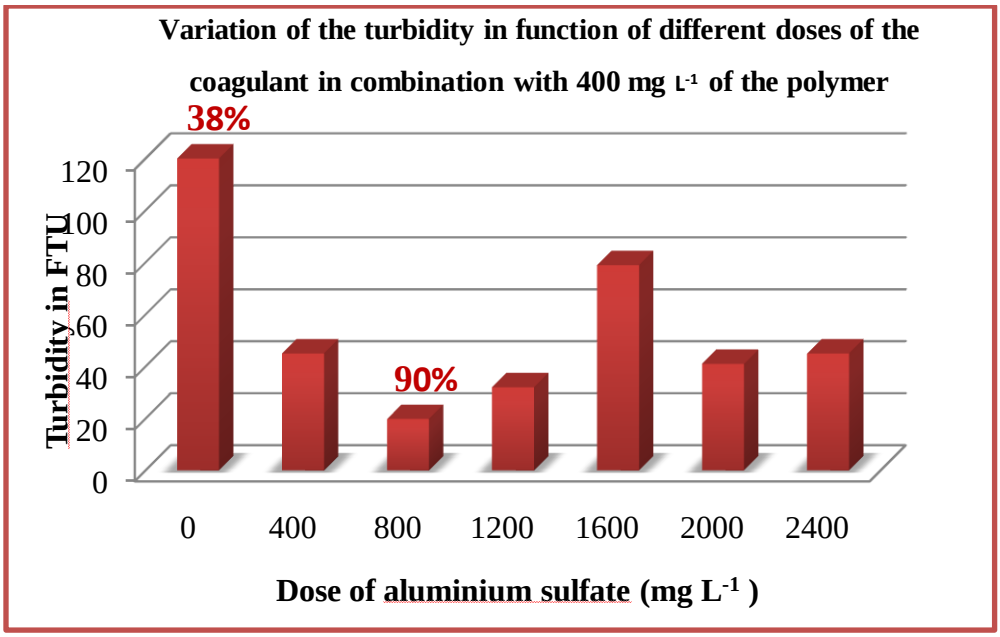

Fig. 1: Variation of the turbidity in function of different doses of the coagulant in combination with 400 mg L^{-1} of the polymer

The whey is adjusted at pH 10; results in the figure 1 show that the turbidity value obtained without aluminum sulfate with a dose of 400 mg L^{-1} of the polymer decreased only 38%, while when combination with different doses of aluminum sulfate was realized, results were improved and noted a significant reduction in turbidity up to 90% (19.8 FTU) with 800 mg L^{-1} aluminum sulfate.

Optimization of the dose of the polymer

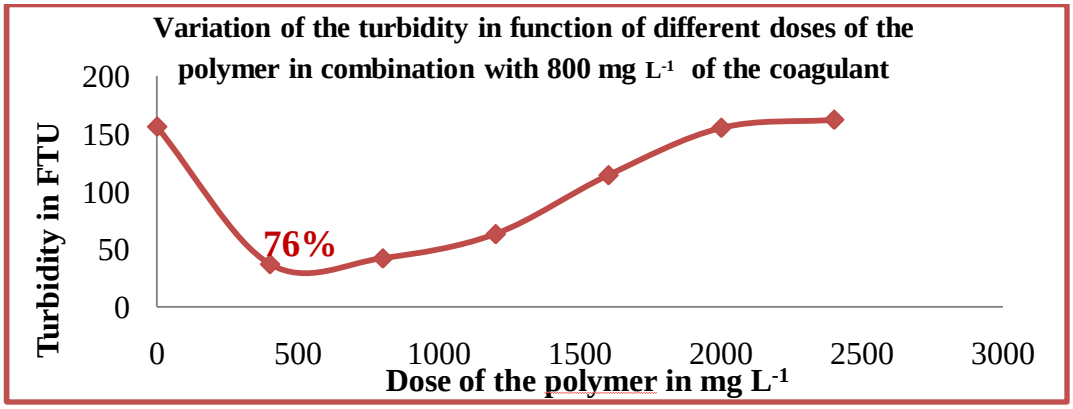

Fig. 2: Variation of the turbidity in function of different doses of the polymer in combination with 800 mg L^{-1} of the coagulant

In this test, the pH is adjusted at 8. Figure 2 shows a reduction of 76% of the turbidity when aluminum sulfate with a dose of 800 mg L^{-1} is in combination with a dose of 400 mg L^{-1} of the polymer. The difference in results between figure 1 and 2 is due to the pH, hence the necessity to study its effect on the coagulant and the flocculent activity.

pH effect

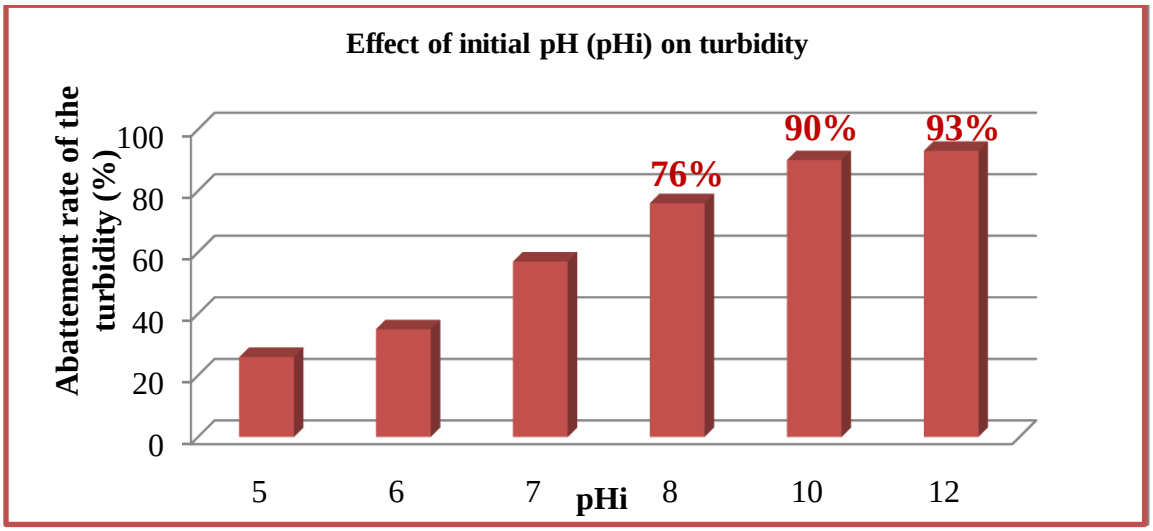

Fig. 3: Effect of initial pH (pHi) on turbidity

By combination 800 mg L^{-1} of aluminum sulfate and 400 mg L^{-1} of the polymer, when pHi of the effluent increases, the turbidity decreases. Thus, significant reduction of the turbidity was observed with a pH over 8 (Figure 3). So pHi 10 is chosen as the best because it's more suitable than the pH 12 for subsequent biological treatment.

The study of the variation between pHi and pHf after adding the optimum dose of coagulant and flocculent shows that the pH always decreases about one degree. This reduction of the pH can be explained by the fact that the addition of aluminum sulfate causes a release of H^+ ions according to the following hydrolysis reaction: $Al^{3+} + n\ H_2O \leftrightarrow Al(OH)_n + n\ H^+$ [16], while the polymer maintains the pH when it is added alone.

Table 2: Characteristics of the diluted coagulated-flocculated whey

Analyzed parameters	Average values	Abatement percentage by coagulation-flocculation
pH	9	-
Turbidity (FTU)	19.8	**90%**
COD (mg L^{-1})	14100	17-40%
BOD_5 (mg L^{-1})	7770	11%
Orthophosphate	0.6	**98%**
Global nitrogen (mg L^{-1})	22.5	**77%**

Table 2 shows the results of physic-chemical treatment. Coagulation-flocculation has reduced various parameters other than the turbidity such as total COD with a variable reduction from 17 to 40 % depending on particle load on the effluent, so more the effluent is rich in particles in suspension, more the percentage of elimination of the particular COD increase. BOD5 decreased by only 11% because this treatment represents no efficiency on the reduction of soluble organic matter. Orthophosphate decreases significantly with 98% giving a value of 0.6 mg L^{-1}. Indeed, in basic or neutral medium, aluminum salts precipitate phosphorous in the form of $AlPO_4$ and then it is removed by the sludge: it's the physic-chemical phosphorous removal. However by combining aluminum sulfate with lime, a reduction of 38.24% of the orthophosphate was observed [7]. A reduction of 77% was observed for the global nitrogen with a value of 22.5 mg L^{-1}, this reduction is resulting from elimination of particular caseins that come from cheese. So for turbidity, orthophosphate and total nitrogen, values have reached the discharge standards, so it remains to eliminate the organic matter, hence the necessity of a secondary biological treatment.

Biological treatment essays

First trial of the biological treatment

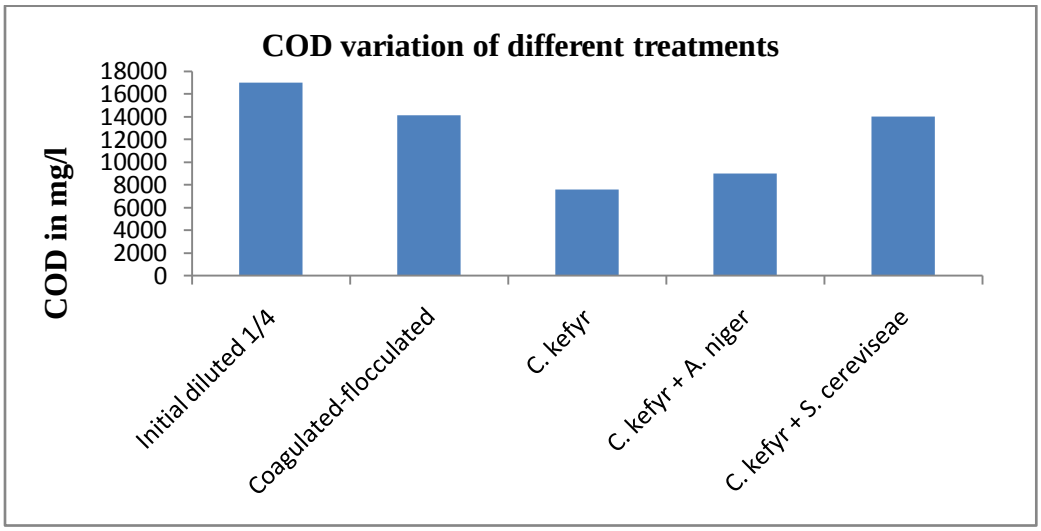

Fig. 4: COD variation of different treatments

The secondary treatment by *C. kefyr* and the mixed culture of *C. kefyr* and *A. niger* noted respectively in the figure 4 a COD reduction of 46% and 36% after 5 days giving final values of 7600 and 9000 mg L^{-1}. It was found that *A. niger* has a little delayed the activity of *C. kefyr* similarly, the treatment with *C. kefyr* and *S.cereviseae* noted no reduction in COD, and this can be explained by a competition between these two strains. *S.cereviseae* inhibited the activity of *C. kefyr*.

Second trial of the biological treatment
Monitoring of the biomass and the COD for the treatment by Candida kefyr

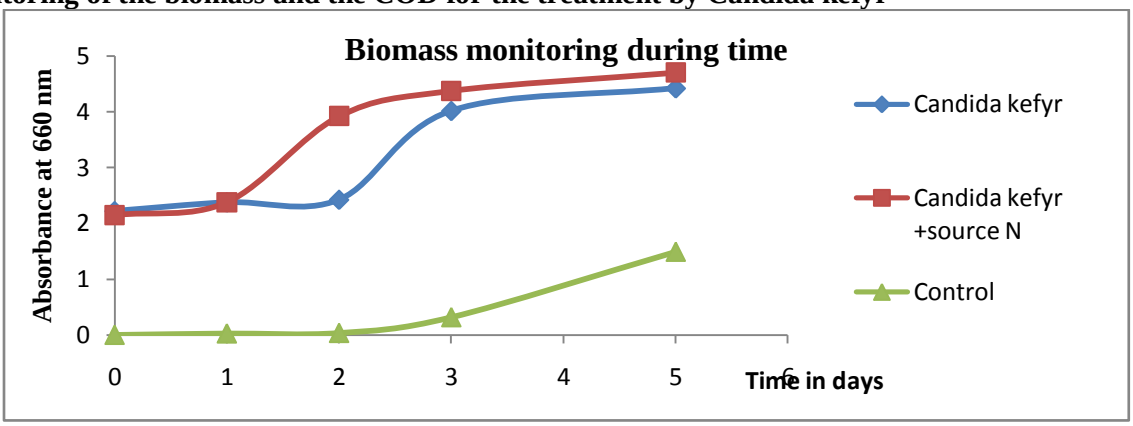

Fig. 5: Biomass monitoring during time using *Candida kefyr*

As shown in the figure 5, the lag phase of *C. kefyr* lasts between one and two days. During this phase the biomass synthesized the ß-galactosidase which allows the consummation of lactose. *Candida kefyr* enriched by a nitrogen source, has rapidly come into the exponential phase providing more biomass than that obtained when *C. kefyr* is unenriched. In both cases, exponential phase lasted 1 day then the biomass go into the stationary phase and could be explained by a limitation of energy source or carbon source in the form of lactose while the other carbon source available in the media will be the glucose and the galactose (issued from the transformation of lactose by ß-galactosidase). In this phenomenon, where there will be a change in the enzymatic material to use the glucose and the galactose, and as the COD remains high on the last day of the treatment, this verifies that a high amount of organic matter as a substrate is still present in the media. As for the control, endogenous bacteria are negligible because the coagulation-flocculation removes insoluble materials such as microorganisms. After 2 days, biomass goes into exponential phase with a very slow growth.

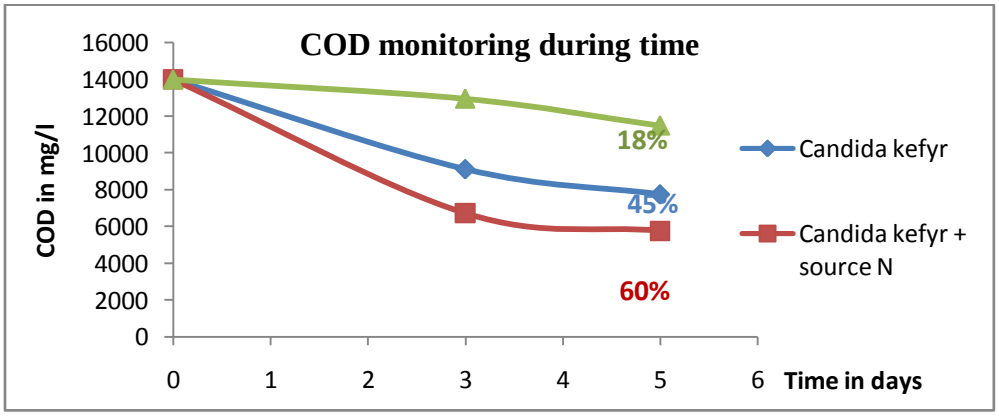

Fig. 6: COD monitoring during time using *Candida kefyr*

According to figure 6, from the third day of the treatment, the COD starts to decline as biomass growth slows. On the fifth day, treatment by *Candida kefyr* decreased the soluble COD of 45% compared to the coagulated-flocculated effluent giving a final value of 7750 mg L^{-1}, but the treatment with *C. kefyr* enriched with nitrogen source showed a reduction of 60% of the soluble COD with a value of 5760 mg L^{-1}. while endogenous bacteria have reduced the COD with only 18% with a value of 11500 mg L^{-1}. Indeed, the reduction in COD can be explained by the use of organic matter present in the media by microorganisms to meet their energy needs required for cellular biosynthesis reactions in the presence of

oxygen [17]. However a physico-chemical treatment followed by a biological one with *Pseudomonas fluorescens* took 20 days to reduce the COD of only 57.35% [7].

Monitoring of the biomass and the COD for the treatment by the mixed culture

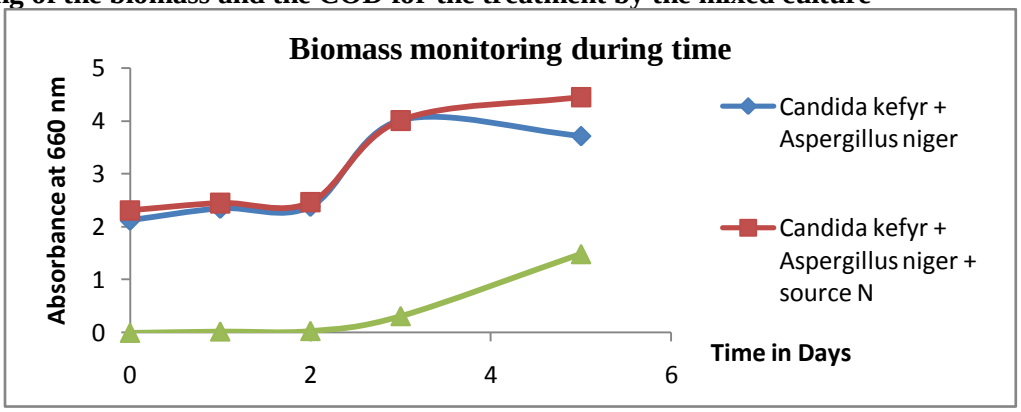

Fig. 7: Biomass monitoring during time using *Candida kefyr* and *Aspergillus niger*

Biological treatment by mixed culture of *Candida kefyr* and *Aspergillus niger* shows the results in figure 7 and 8. The results in the figure 7 show that in presence or absence of a nitrogen source, the growth of double culture is the same for the lag and exponential phase. With no nitrogen, growth decreases after the exponential phase and this is due to the limitation in nitrogen source. When nitrogen is in the media, biomass goes into stationary phase due to limitation in carbon source in the form of lactose.

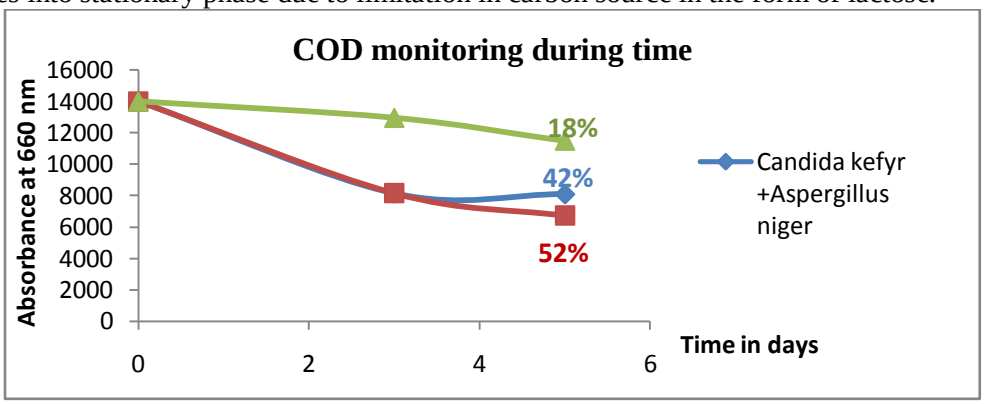

Fig. 8: COD monitoring during time using *Candida kefyr* and *Aspergillus niger*

According to figure 8, in both cases of the mixed culture, the reduction of the COD obtained until the third day is 42% compared to the effluent coagulated and flocculated, giving a value of 8150 mg L^{-1}. At the end of the treatment, this mixed culture with a nitrogen source, reduced the COD to 52% noting a value of 6720 mg L^{-1}, whereas in the presence of nitrogen a slight reduction occurs to 8100 mg L^{-1}. Note that reductions of soluble COD are lower than those obtained when *Candida kefyr* is alone, and this shows that the mixed culture did not improve treatment and competition has taken place between these two strains. *A. niger* has a little delayed the activity of *C. kefyr*. So the best treatment to reduce the COD is obtained by *Candida kefyr* enriched with a nitrogen source. Overall, when the treatment is preceded by a physico-chemical treatment, it can reduce the COD of 66% compared to the initial diluted effluent. Contrary to a study that showed that the addition of a nitrogen source (0.01 and 0.015% w/v) to the treatment by *Candida kefyr* reduced its cellular growth and lactose consumption [9].

DBO_5 reduction

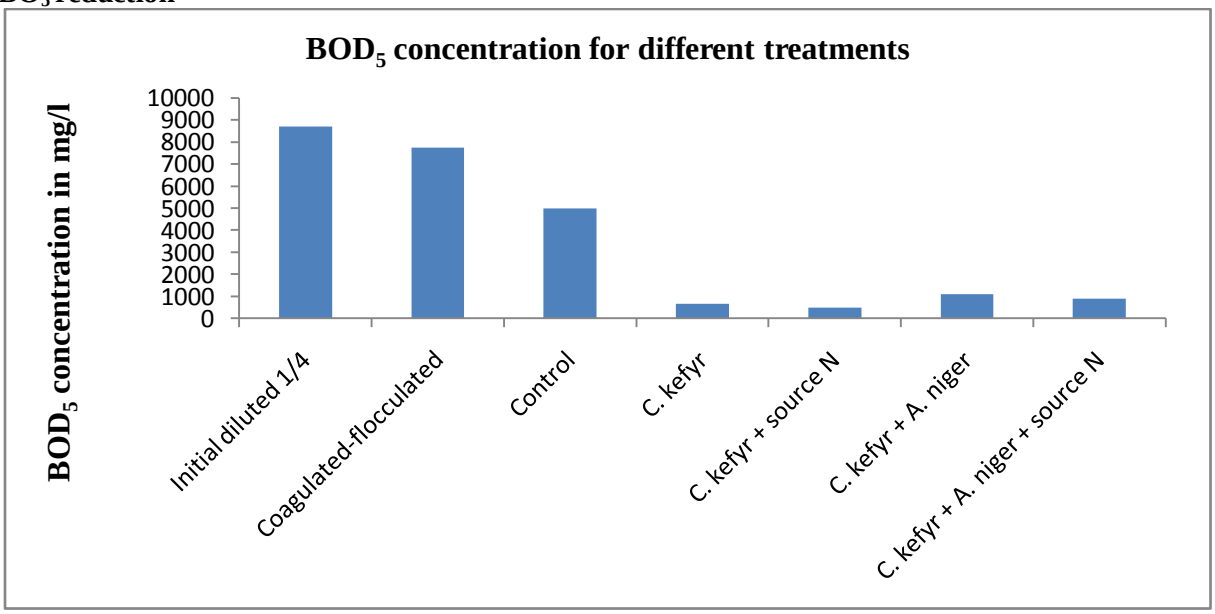

Fig. 9: DBO_5 concentration for different treatments

The physico-chemical treatment followed by a biological one by *Candida kefyr* decreased BOD_5 of 92% compared to the initial diluted effluent giving a value of 679.4 mg L^{-1}. in case of nitrogen enrichment, the reduction rate increases up to 94% noting 506.8 mg L^{-1}. Despite the competition between *C. kefyr* and *A. niger*, a significant decrease of 87% was observed noting a value of 1106 mg L^{-1}. Similarly, in the case of nitrogen enrichment, a small increase in the rate of reduction is marked up to 89% and record a value of 897.2 mg L^{-1}. So the treatment realized giving best results for BOD_5, is still the treatment realized by *Candida kefyr* enriched with nitrogen source (Figure 9).

Total nitrogen reduction

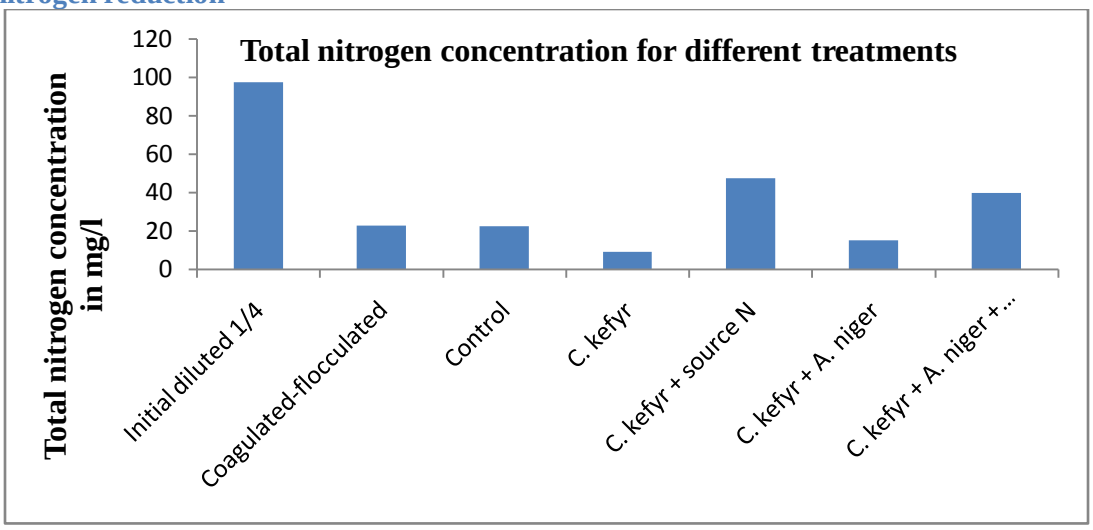

Fig. 10: Total nitrogen concentration for different treatments

Results of the total nitrogen reduction are shown in the graph of figure 10. Coagulation flocculation that decreased total nitrogen to 22.5 mg L^{-1} (reduction of 70%), and then the biological treatment by *Candida kefyr* occurs to reduce total nitrogen for about 60% and giving a final value of 9 mg/l. So overall, a physic-

chemical treatment followed by a biological one by *Candida kefyr* reduced total nitrogen of 91% from initial diluted effluent. This reduction can be explained by the degradation of soluble whey proteins and ammonium by microorganisms in order to meet their energy needs and then the synthesis of cellular proteins. When *Candida kefyr* is enriched by ammonium sulfate (1g L^{-1}), total nitrogen notes a high value of 47.3 mg L^{-1} at the end of the treatment. This concentration is due to the addition of a nitrogen source in the form of ammonium (≅ 266 mg L^{-1}) consumed by *C. kefyr*. In mixed culture, the concentration of total nitrogen noted a value of 15 mg L^{-1} and therefore an abatement rate of only 33% compared to the coagulated-flocculated effluent. This may be due to the competition which takes place in two cultures. So the overall rate reduction by the physic-chemical treatment followed by biological one by the mixed culture noted a percentage of 84% compared to the initial diluted effluent. Similarly, in case of addition of ammonium sulfate, total nitrogen noted a high final value of 39.8 mg L^{-1}. the best treatment obtained concerning nitrogen reduction is that realized by *Candida kefyr*, but due to benefits presented by the nitrogen source, its recognized to enriched it by the ammonium but with a reduced concentration of ammonium sulfate added initially in order to obtain a final concentration conforming to discharge standards.

Orthophosphate

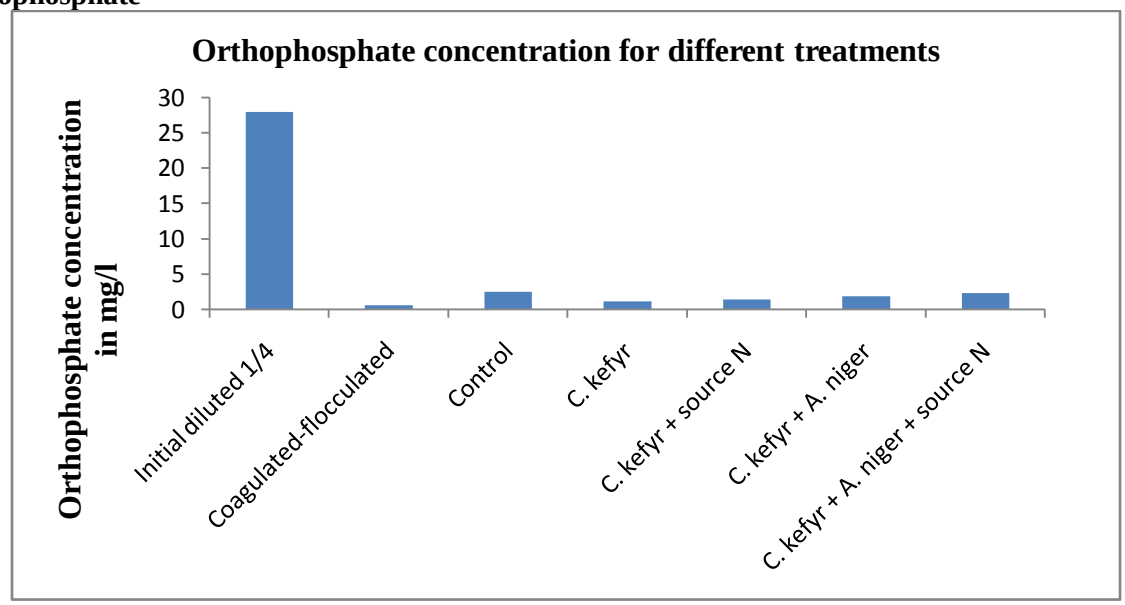

Fig. 11 : Orthophosphate concentration for different treatments

Figure 11 shows that with all biological treatment preceded by a physic-chemical treatment, a moderate augmentation of orthophosphate was observed compared to the coagulated-flocculated effluent, and can be explained by the degradation of the organic phosphorous of the whey during the 5 days of the treatment. Despite this augmentation, values remain respectful to the discharge standards and abatement rates remain above 90% comparing to the initial diluted effluent. The physico-chemical treatment followed by a biological one by *Candida kefyr* enriched by a nitrogen source, chosen as the treatment giving the best results, noted a concentration of orhtophsophate about 1.48 mg L^{-1} and therefore a reduction rate of 95% comparing to the initial diluted effluent.

APPLICATIONS

All methods applied in this work can be used for different water and wastewater treatment analysis. This study shows a simple and economic method for dairy industrial wastewater treatment that could be applied on industrial scale.

CONCLUSIONS

Discharges of cheese industries constitute a real threat to the environment because of the large volumes of whey generated and its high load with organic material. For this, the whey processing is therefore a responsibility to seek a simple and inexpensive method that can be applied on an industrial scale. For this purpose, this study includes a coupling of a physic-chemical and a biological treatment of diluted acid and sweet whey, in order to study the effectiveness and the ability of this treatment to degrade organic matter and reach the possible discharge standards. Coagulation-flocculation optimized by aluminum sulfate and the polymer Zetag 48 showed their effectiveness in turbidity, orthophosphate and total nitrogen with respective reductions of 90%, 98% and 77%. When this treatment is coupled to a secondary biological treatment by *Candida kefyr* enriched by a nitrogen source (1g/l of ammonium sulfate), significant reductions of the COD and the BOD_5 are observed respectively of about 66% and 94% comparing to the initial diluted effluent during 5 days. Other than the treatment of dairy effluents, several recovery processes are possible in order to avoid the huge discharges from dairies. In this sense, Hilan and al. in 2000 [18], proposes to use whey as a supplement to cattle feed, and it can be used in feed for diabetic patients or subjects with bas nutrition [19]. On the other hand, whey can be used as a culture medium to produce biomass [8] and several products with high industrial values: lactic acid, vitamins (B2, B12), enzymes (protease, amylase, cellulose and ß-galactosidase) and fat [20].

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