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Abstract

Kefir grains are a microbial consortium of different genera of bacteria and yeasts. In this study, the performance of Tunisian Kefir grains during the biological treatment of a mixture of Gouda cheese whey and white wastewaters (GCW) in ratio 1:1 with very high organic matter concentration is investigated. The biological

process was evaluated and optimized through the response surface methodology. Under the optimum conditions, Kefir grains concentration of 1.02%, temperature at 36.68 °C, and incubation time of 5.14 days, the removal efficiencies of COD, PO_4^{3-}, and NO_3^- were 87, 37.48, and 39.5%, respectively.

Interestingly, the reusability tests of the grains proved not only their high resistance to harsh environmental conditions but also their great potential for more practical applications. Particularly, different strains were isolated from the grains and identified as *Kluyveromyces marxianus, Lactoccocus lactis, Lactobacillus kefiri,* and *Bacillus spp*. using 16S rDNA sequence analysis and rep-PCR fingerprinting. At the biological level, the raw GCW (RGCW) has a negative impact on the Hordeum vulgare both on seed germination, and on the growth parameters of seedlings. Interestingly, after Kefir grains treatment, the treated GCW (TGCW) allow a seedlings growth and germination rate similar to those soaked in water.

Introduction

In recent years, dairy and cheese industries have been known among the fastest growing agrofood companies worldwide. These industries produce a significant volume of different liquid effluents especially wastewaters (Martínez-Suller et al. 2010) and valuable byproducts mainly whey (Panesar et al. 2007). The produced volume and the chemical composition of these effluents are significantly variable and depend largely on the different stages used during the making process and the final products (Pattnaik et al. 2007; Carvalho et al. 2013). Wastewaters, with low organic loads ranging from 2.5 to 3 L per L of processed milk on average (Singh et al. 2014), mainly contain milk losses and washing water from equipment sections, bottles, and tanks (Carvalho et al. 2013). Globally, the volume of cheese whey (CW), with an average value around 0.9 L generated from 1 L of processed milk (Nicolás et al. 2019), accounts for about one-third of the total effluents of cheese factory (Chatzipaschali and Stamatis 2012). Generally, it comprises 85–95% of the milk volume and retains about 55% of the milk constituents (Ryan and Walsh 2016). Besides, it is characterized by relatively high concentrations of biodegradable organic matter (Chatzipaschali and Stamatis 2012).

From a valorization point of view, approximately 50% of 190 million tons of whey produced worldwide every

year is processed for effective purposes in the medical, pharmaceutical, agroindustrial (Baldasso et al. 2011; Ryan and Walsh 2016), and agricultural fields (Prazeres et al. 2012, 2016). Recently, research on the production of kefir, a natural probiotic beverage, from CW fermentation with Kefir grains has shown exponential interest in its potential effective benefits to human health (Rosa et al. 2017). These grains are irregularly shaped hard granules with a yellowish-white color which resemble miniature cauliflower blossoms (Leite et al. 2012; Rosa et al. 2017). They are a symbiotic association of bacteria especially Lactobacillus, Leuconostoc, Lactococcus, and Acetobacter, and yeasts mainly Kluyveromyces , Saccharomyces, Candida, and Torulaspora. These bacteria and yeasts are naturally immobilized in a matrix of proteins and heteropolysaccharide “kefiran” and their different combi-nations at the species level generally characterize each local product (Zanirati et al. 2015). According to the literature, the use of Kefir grains in agro-food industries is soaring given the fact that the grains can be successfully produced on a large scale in a low-cost culture. They also exhibit excellent resistance to physical and chemical stresses (Magalhães et al. 2010; Londero et al. 2015; Plessas et al. 2017). Yet, in the environmental field, there are no reports on the biological treatment of dairy wastewater using Kefir grains. In addition, our previous research (Elleuch et al. 2020) was, to our knowledge, the first study to report on the effective and low-cost biological pretreatment of wastewater (landfill leachate) using Kefir grains with its high organic matter content and toxicity. Under the optimum conditions, the overall removal rates of TOC, COD, NH4 +-N, and PO_4^{3-} were 93, 83.33, 70, and 88.25%, at an initial COD concentration of 24,000 mg/L, respectively. Besides, the grains exhibit excellent reusability and resistance to harsh environmental conditions (Elleuch et al. 2020).

In Tunisia, the dairy and milk processing sector includes 45 companies, and the cheese subsector is composed of 25 enterprises. The dairy production approximately reached 1.2 billion liters in 2014 with a daily processing capacity of about 3.8 million liters and an average of0.5 million liters are processed daily for cheese making (APII 2014). Consequently, different dairy effluents with a high organic matter content are produced and their disposal without treatment and valorization represents a serious environmental problem causing considerable economical losses (Kasmi 2016). Over the past decades, many studies have focused on the treatment of dairy wastewaters using different biological and physicochemical methods; only a few studies, however, have dealt

specifically with the CW treatment without biotechnological valorization strategies.

Recently, it has become worthwhile research to turn to the strategic environmental challenge for effective treatment and advanced valorization technologies of different wastes generated from the entire dairy chain into economic incentives. In this context and from an economic point of view, recent studies have highlighted the potential reuse of dairy wastes for nutritive components production (Kasmi et al. 2017a), isolation and selection of lactic acid bacteria for their antimicrobial activities against different pathogenic bacteria causing nosocomial infections (Ghodhbane et al. 2016), and low-cost lactic acid bacteria growth media production (Kasmi et al. 2018). On the other hand, Tsolcha et al. (2018) described the efficiency of a Leptolyngbya-based microbial consortium for biological treatment of second CW effluent and biodiesel production. Furthermore, Paçal et al. (2019) reported the effective treatment of CW wastewater and biogas production using anaerobic dynamic membrane bioreactor. In addition, anaerobic digestion based on the biological reduction of organic compounds to biogas is proposed as ecofriendly technology for industrial dairy wastewater (Mainardis et al. 2019; Charalambous et al. 2020;Treu et al. 2019). In Tunisia, the reuse of treated wastewater as an alternative water source in agriculture has been growing rapidly since 2013 (Sdiri et al. 2018). Interestingly, Toumi et al. (2015) reported that treated dairy wastewaters have the potential to be reused as biofertilizers. Furthermore, recent results con- firmed that the treated dairy wastewaters, following the re- quired Tunisian legislation, have effectively improved the growth parameters of wheat (Sioud et al. 2016) and the bio- mass production of olive plants of the variety “Chemlali” (Sdiri et al. 2018). In In this research, the performance of the Tunisian Kefir grains process of a mixture of Gouda cheese whey and white wastewaters (GCW) was investigated using Box-Behnken de- sign (BBD). In addition, different strains of yeasts and bacteria were isolated from the grains and identified using 16S rDNA sequence analysis and rep-PCR fingerprinting. Furthermore, the impact of treated GCW (TGCW) with different dilution on morphophysiological parameters: germination rate, fresh weight, shoot and root lengths, and chlorophyll contents of the seedlings of Hordeum vulgare, in comparison to raw GCW (RGCW) and control (water) was evaluated.

Materials and methods

Samples characterization

In this study, the selected wastes, CW, and white wastewaters (WW) were collected from a regional cheese-making factory located in the industrial zone of Ben Arous, Tunisia, and stored at – 20 °C to avoid their acidification and chemical composition modification. CW obtained from the manufacturing of Gouda cheese and WW, mainly rich in milk and water, is generated after the procedure of equipment washing. After preliminary experiments, a mixture of these two Gouda cheese wastes (GCW) in ratio 1:1 was selected to be treated by a biological process with Kefir grains. The physicochemical characterization of the raw CW, WW, and GCW is presented in Table 1. The COD and pH values of GCW were 46.080 g/L and 4.36, respectively. In general, the physicochemical properties of CW were characterized by high variability, and a COD range of 49.87–78.73 g/l was reported in the study of Mainardis et al. (2019) while a COD value of 77.5 g/l was described in the work of Treu et al. (2019).

Kefir grains

In this study, the grains were prepared on CWaccording to the method of Magalhães et al. 2010 with slight modifications. The grains 10% (w/v) were inoculated into fresh CW at 25 °C for 24 h without stirring. The experiment was repeated three times, and the activated grains were used for further analyses. The reusability of the grains was tested as described by Elleuch et al. (2020).

Isolation and identification of microorganisms from Tunisian Kefir grains

Free Kefir cells were isolated from activated grains as follows: 10 g ofthe grains sample were suspended in 27.6mL ofsterile Ringer solution (Sigma-Aldrich, Saint Luis, MO, USA) and homogenized using a Stomacher 400. Serial dilutions were used for microbial enumeration and isolation on different me- dia. The following microbial species were enumerated: lactobacilli on Man Rogosa Sharpe (MRS; Lab M®, Heywood, Lancashire, UK) supplemented

with 0.025 g/mL of Delvocid (Sigma) and cultivated at 30 °C for 48 h and yeasts on W.L nutrient agar (Lab M®) nutrient agar supplemented with 0.05 g/mL of tetracycline (Sigma) and incubated at 25 °C for 48 h. Results were expressed as the decimal logarithm of colony-forming units (CFU) per gram of Kefir grains (± standard deviations). The isolated bacteria and yeasts were were further purified, grown in MRS and YPD (Lab M®) broth, respectively, and stored at − 20 °C with 20% glycerol.

Rep-PCR

The genomic DNAs of the different isolated bacteria and yeasts were extracted as described by Cocolin et al. 2001. Then, rep-PCR fingerprinting was carried out using the primer (GTG)5 (5′ - GTGGTGGTGGTGGTG-3′) according to Dal Bello et al. 2010. The obtained products were visualized under ultraviolet light, and the resulting profiles were determined by a digital image capturing, using a CCD UVI pro Platinum 1.1 (Eppendorf). The BioNumerics 4.6 software package was used to analyze the rep-PCR fingerprints. Group differences in the microbial community structure of Kefir grains were performed using unweighted pair group method with arithmetic mean (UPGMA), and the Pearson's correlation coefficient was used to assess the similarity between profiles.

16S rDNA gene sequencing of Kefir grains isolates

Representative microbial isolates of subcluster at 70% of similarity were identified by sequencing the partial rRNA amplicon. The 16S rDNA was amplified from the bacterial strains with the primers FD1 (5′ - AGAGTTTGATCCTG GCTCAG-3′) and RD1 (5′ -AAGGAGGTGATCCAGCC-3′) as described by Weisburg et al. (1991). For the yeasts, the Internal transcribed spacer (ITS) region (ITS1‑5.8S-ITS2) was amplified with the two primers ITS1 (5′ -TCC GTA GGT GAA CCT TGC GG-3′) and ITS4 (5 ´-TCC TCC GCT TAT TGA TAT GC-3′) according to Korabečná et al. (2003).

Biological process

The optimization of Kefir grains treatment parameters for GCW was carried out by using BBD based on response sur- face methodology with the statistical software design expert Version 10.0.6 (Stat- Ease Inc., MN, USA). Fifteen experiments were conducted with three independent factors: tempeature (X1), incubation time (X2), and Kefir grains concentration (X3) at three different levels and under different conditions to evaluate their interactions and the importance of their effectiveness on the removal of COD (Y1), PO_4^{3-} (Y2), and NO_3^- (Y3), selected as responses (Table 2). The biological process was carried out at a small-scale system under non aseptic conditions, and the experiments were performed with 50 mL of GCW inoculated with Kefir grains in a 250-mL Erlenmeyer flask and incubated without any pH adjustment and agitation. The evaluated response Y was calculated using the following equation:

$$Y = a_0 + \sum_i a_i X_i + \sum_{ij} a_{ij} X_i X_j + \sum_{ii} a_{ii} X_i^2$$

where Y is defined as the evaluated response for the removal efficiency of pollutants, a0, ai (i = 1,2,3) aii (i = 1,2,3), and aij (i = 1,2,3; j = 1,2,3)are the model coefficients and Xi and Xj the coded independent variables. NemrodW software (LPRAI version 2000) was used to analyze the variance (ANOVA) results and perform form the response surface curves.

Analytical methods

Chemical analyses of the raw and treated cheese effluents were performed using standard methods described by Rodier et al. 2009. PO_4^{3-} and NO_3^- were determined using an ion chromatography Metrohm 761. Conductivity/pH meter con- sort C860 was used to determine the pH and the conductivity of the samples. The turbidity was determined by using a turbidimeter (WTWTurb 555). The different analyses were per- formed in triplicate.

Evaluation of treated GCW on seedlings growth of barley

The seeds of barley (*Hordeum vulagre*) were sterilized with HgCl2 solution (0.1%) and thoroughly washed with sterile distilled water. Five uniform seeds were placed in 90-mm Petri dishes lined with a filter paper moistened with 15 mL of one of the different dilutions (25, 50, and 100X) of RGCW and TGCW. Three replicates were carried out for the different samples, including the control with distilled water (H2O), and the Petri dishes were incubated in a dark incubator at 20 ± 2 °C for 3daysthenin a photoperiod (16hlight/8hdark) for 1 week. The germination rate (GR) was calculated ac- cordingtoKomilis et al. 2005, and the different growth parameters, leaf and root lengths and fresh weight, were determined after 10 days of germination of the seeds with regular observation at an interval of every 24 h.

Determination of chlorophyll content

Fresh leaves (0.1 g) were homogenized with 10 mL 80% chilled acetone in a prechilled mortar and pestle. Concentrations of chlorophylls a (Chl a), b (Chl b), and total chlorophyll were calculated according to Arnon 1949.

Results and discussion Microbiological

Microbiological analysis of Tunisian Kefir grains

Different genera and species of yeasts and bacteria have been isolated and identified from Kefir grains collected from different locations (Garofalo et al. 2015;Dertli and Çon 2017;Gut et al. 2019). Interestingly, it has been proved that their complex microbial composition is ex- tremely variable and depends mainly on geographical regions and culture conditions (Marsh et al. 2013; Zanirati et al. 2015;Arslan 2015). In this section, Tunisian Kefir grains were analyzed microbiologically to identify the predominant microbial populations. The MRS counts were 5.04 ± 0.57 Log CFU/g while yeasts were present in the grains at 6.25 ± 0.05 Log CFU/g. A total of 81 isolates (54 bacteria and 27 yeasts) were subjected to rep-PCR fingerprinting technique in order to group them at genus/species level. Later on, 11 bacteria and 4 yeasts were chosen as representatives of each subcluster obtain- ed (70% of similarity) and identified as the bacteria Lactococcus lactis, Lactobacillus kefiri, and Bacillus spp. (Fig.

199 1a)and the yeast Kluyveromyces marxianus (Fig. 1b). These findings are in accordance with previous observations
200 of Garofalo et al. (2015). It is worth noticing, in this vein, that several studies have highlighted the successful use
201 of these genera of bacteria (Kasmi et al. 2017a, b; Ghasemi et al. 2017; Al-Wasify et al. 2017) and the the yeast
202 Kluyveromyces marxianus (Yadav et al. 2014) for the biological treatment of dairy effluents.
203
204 **Kefir grains process**
205 **Optimization of Kefir grains process using BBD**
206 The preliminary experiments showed the significant effects of the culture conditions especially temperature (X1),
207 incubation time (X2), and Kefir grains concentration (X3) of Kefir grains on the removal of COD, PO_4^{3-}, and NO_3^-.
208 On the basis of these findings, BBD was specifically selected to evaluate the interactions between these factors
209 and the importance of their effectiveness on the removal of COD (Y1), PO_4^{3-}Y2), and NO_3^- (Y3). Table 3 shows the
210 values of the independent factors and the predicted and experimental values of the responses.
211
212 **Analysis of the experimental data**
213 The optimization of Kefir grains process by BBD includes the study of the response of the designed combinations
214 of factors, the estimation of the different coefficients, the response prediction of the fitted model and the testing
215 of the significance, and adequacy of the model. Firstly, the ANOVA tests were conducted for the three studied
216 responses: COD (Table 4), PO_4^{3-} (Table 5), and NO_3^- (Table 6) removal rates. In statistics, coefficients with P value
217 less than 5% show that model terms are significant whereas, the coefficients with P value more than 5% are
218 considered as insignificant.
219 For COD removal rate (Y1), the obtained results indicated that the three studied factors are insignificant (Table4)
220 and the response was calculated using the following equation:Y1 ¼ 68:52 Concerning the PO43− removal
221 efficiency (Y2), it is evident from Table 5 that only the incubation time fac- tor (X2) is positively significant (P value
222 < 5%), and the different interactions have no significant effect.
223 Consequently, the mathematical model is obtained as follows:

$Y_2 = 32.6 + 4.08\ X_2$

In contrast, as shown in Table 6, the temperature is more significant than the grains concentration and the incubation time on the removal of NO_3^-. In addition, only the interaction effect between temperature and Kefir grains concentration is significant. Thus, the response Y3 was calculated as follows:

$Y3 = 32.29 + 5.23\ X_1 - 4.26\ X_2 - 2.2\ X_3 + 5.92\ X_{11} + 3.60\ X33 - 5.46\ X_{13}$

As presented in Table 7, the significance and adequacy of the model are confirmed by the variance analysis and Fisher's F test values.

Effect of variables on COD, PO_4^{3-} , and NO_3^- removal rates

The concurrent effect of temperature, incubation time, and Kefir grains concentration onCOD, PO_4^{3-}, and NO_3^- removal efficiencies during Kefir grains process was evaluated (Fig. 2). According to the response surface plots, the maxi- mum removals of COD and NO_3^- were obtained at the low level of Kefir grains concentration. In addition, the increase in incubation time improved the biological treatment, whereas, temperature proved to be an irrelevant factor.

Optimization using desirability functions

The reuse of treated industrial wastewaters with a relatively low concentration on COD and no excessive amount of nutrients especially, nitrogen (N) and phosphorus (P) used as growth factors for plants is a common practice of irrogation in many parts of the world. In this study, RGCW was treated with Kefir grains in or- der to enhance their quality for further application in agriculture. Hence, the main goal of the biological process with Kefir grains is to maximize the removal rate of COD and reduce the rates of PO43− and NO_3^- to the desired concentrations with recalculating the values of responsible factors by using the desirability function approach. According to the BBD results, the maximum predicted values of COD, PO_4^{3-} and NO_3^- are found to be 86.78, 35.95, and 38.76%, respectively, during the biological process with 1.02% Kefir grains at 36.68 °C during 5.14 days without agitation and any pH adjustment. Under these conditions, an additional experiment was performed, and the obtained

249 results showed that the removal efficiencies of COD, PO_4^{3-},and NO_3^- were 87 ± 0.5, 37.48 ± 0.74, 39.5% ± 0.39,
250 respectively. It is clear that Kefir grains can reduce the organic compounds of GCW characterized by a high COD
251 concentration (25,920 mg/L). This is in line with other studies prov- ing that the different organic compounds
252 from CW can be significantly reduced during the biological process with the pure culture of Bacillus sp., coculture
253 of Bacillus sp. with Cupriavidus sp. (Reddy et al. 2019) and mixed culture of the two yeasts Kluyveromyces
254 marxianus and Candida krusei (Yadav et al. 2014). In general, CW contains soluble proteins, lipids, vitamins,
255 mineral salts, and mostly lactose responsible for high BOD and COD content (Saini et al. 2017). Lactose is a
256 disaccharide fermented only by microorganisms ex- pressing both the membrane transporter, lactose permease,
257 and the hydrolytic enzyme β-galactosidase (Grba et al. 2002). Lactococcus lactis strains are homofermentative
258 bacteria ferment lactose into pyruvic acid, which is, then, reduced to lactic acid by the reducing power previously
259 produced in the form of NADH. While, Lactobacillus kefiri strains classified as heterofermentative lactobacilli
260 produce acetate, carbon dioxide, ethanol, and/or acetic acid in addition to lactic acid as the end product of
261 fermentation-phosphoketolase pathway (Bintsis 2018). Additionally, various metabo- lites are released from
262 proteins and lipid fractions pres- ent in cheese whey through many enzymatic reactions (Burgain et al. 2014). On
263 the other hand, Kluyveromyces marxianus is lactose-fermenting yeast with useful physiological features such as
264 high growth rate and thermotolerance. The coregulated genes LAC4 and LAC12 encoding β-galactosidase and
265 lactose per- mease, respectively, are responsible for lactose uptake and hydrolysis to glucose and galactose (Saini
266 et al. 2017). So far, several studies have reported various biological processes of synthetic dairy wastewater
267 under asep- tic conditions while a few studies have focused on the biological treatment of real wastewater under
268 non- aseptic conditions. In this work, the biological treatment of a mixture of two real dairy effluents with very
269 high organic matter concentration was studied under non- aseptic conditions. Tsolcha et al. (2018) have studied
270 the removal of organic and inorganic compounds from dairy wastewater at dilution ratio (8:100) by a mixed
271 microbial consortium. The effluent contains initial pol- lutants concentrations lower than those presented in this
272 research and after biological treatment under non-aseptic conditions, the removal rates of COD, NO_3^-,and PO_4^{3-}
273 were 93.5, 54.5, and 83.2%, respectively.

Reusability tests of Kefir grains during GCW treatment

Currently, the level of reusability of immobilized microorganisms is an important issue for practical environmental applications. Therefore, the reusability tests of Kefir grains during GCW treatment were studied for two cycles and at the end of the second cycle, the removal rate of COD, PO_4^{3-},and NO_3^- were 82.6, 30.8, and 32.3%, respectively (Table 8). Overall, Kefir grains process can be regarded as an economical and ecofriendly process with no secondary pollution effects since the produced biomass can be reused and allows efficient removal of pollutants. Its advantages include feasi- bility, practicability, reliability, simplicity, and absence of un-pleasant odors. Compared with free or immobilized strains, Kefir grains

display many features that make it particularly suitable for industrial dairy applications under non-aseptic conditions. Indeed, Kefir grains are a natural consortium containing a unique, complex, and stable microbial community with the predominance of lactic acid bacteria, acetic bacteria, yeasts, and fungi (Laureys and De Vuyst 2014; Garofalo et al. 2015). All these microorganisms exist in a state of symbiotic equilibrium in a natural exopolysaccharide and a pro- tein matrix (Gao and Zhang, 2019). Interestingly, Kefir grains are a natural mixed starter culture commercially used in food industries for the production of cheese, bread (Plessas et al. 2017), and various probiotic beverages with interesting healthy properties (Gao and Zhang 2019). In addition, they can be stored for long periods (Fiorda et al. 2017). Besides, the freeze-dried culture retains a high survival rate and shows good metabolic activity and fer- mentation efficiency which is important for their industrial applications (Prado et al. 2015). Furthermore, their recovery is very easy, and CW has been frequently used in large scale as a low-cost substrate for growth and biomass production under non-aseptic conditions (Magalhães et al. 2010; Plessas et al. 2017). Regarding wastewater treatment, the use of Kefir grains has been studied for the first time in biological pre-treatment of landfill leachate with its high organic matter content and toxicity, and the grains exhibit excellent reusability and resistance to harsh environmental conditions (Elleuch et al. 2020). Yet, during the biological process with free microbial cells dispersed throughout the mixed culture medium and raw effluent, it is practically very difficult to harvest them for other cycles of reuse (San et al. 2014). Additionally, the culture

of free or immobilized strains generally requires commercial media under specific and sterile conditions which increases the process costs and therefore limits their application and reusability in large- scale and industrial-scale systems. Generally, the direct use of the biological process presents some disadvantages such as a large amount of sludge generation, slower treatment time, and unintended inhibition with an output of noisome smell (Gogate et al., 2020). In this study, the performance of Kefir grains for the cheese wastewater treatment was investigated at a small-scale system; therefore, it is difficult to discuss the disadvantages of the process.

Impact of GCW on the morpho-physiological parameters of Hordeum vulgare

The impact of RGCW and TGCW with their different dilutions (25, 50, and 100X) on barley was studied. The different parameters of the seedlings growth, germination rate (GR), fresh weight (FW), and shoot and root lengths, were evaluated in comparison to control (Fig. 3). It is noted that the different effects of RGCW and TGCW on Hordeum vulgare seedlings growth traits may mainly depend on GCW quality and dilution. RGCW completely inhibits seed germination (data not shown). These results confirm the findings from previous studies which focused on the evaluation of the effects of cheese and dairy effluents on the germination and growth of crops (Prazeres et al. 2014; Toumi et al. 2015; Sioud et al. 2016; Abou-Dahab et al. 2019). According to these researchers, the negative effect of RGCW may be attributed to the toxicity caused by the different amounts of organic and inorganic compounds present in the effluent. Figure 3 shows that the Kefir grains process improved the quality of GCW and all the studied parameters were significantly better with TGCW than those obtained with RGCW (Fig. 4). The results of TGCW at 50 and 100X are similar.

Subsequently, the effect of TGCW 50X on the content of leaf photosynthetic pigments was studied. Regarding the total chlorophyll content, similar results were obtained between the different seedlings either soaked with RGCW or TGCW (Fig. 5a).

Similarly, previous research showed that the interaction between olive mill wastewater-compost and foliar application with ZnSO4 increased the growth parameters of H. vulgare, while no significant differences in total chlorophyll content were observed (Abdel-Ati and Eisa 2015). Regarding the chlorophyll b, RGCW and TGCW

have a negative impact compared to control plants (Fig. 5c). Concerning chlorophyll a, barley seedlings registered different results between RGCW and TGCW (Fig. 5b).

Interestingly, the highest chlorophyll a content was obtained with TGCW. Similarly, Sdiri et al. (2018) reported a sig- nificant difference between the results of the effect of dairy wastewater on chlorophylls contents of olive leaves and indicated that treated wastewater improved significantly chlorophyll a content. Generally, treated wastewater is a valuable source of water and nutrients which are the major factors enhancing chlorophyll contents (Ashrafi et al. 2016). According to Croce and van Amerongen (2014), the differ- ence in chlorophyll a and chlorophyll b contents could be related to the fact that chlorophyll a acts the first in the light- harvesting complexes and contrarily, chlorophyll b, cannot act as the primary donor within the reaction centers. This study is a first attempt to investigate the performance of Kefir grains for cheese wastewater treatment at a small- scale system under non-aseptic conditions. Removal rates of COD, $PO_4^{3-}$and NO_3^- reached 87, 37.48, and 39.5%, respectively under the optimum treatment conditions. Therefore, this study can be a starting point for further re- search to be performed gradually at lab-scale system, pilot- scale system, and full-scale industrial in wastewater treatment plants to establish the best operating parameters in terms of pollutants removal from cheese wastewaters. Interestingly, the treated effluent has a positive effect on barley seedlings growth parameters and chlorophyll a content, and further tests should be executed in order to use treated effluent as liquid fertilizer by mixing it with soil after appropriate dilution.

Conclusion

In the present study, a new practical approach for biological treatment with Kefir grains of a mixture of GCW was successfully developed. The BBD was applied to deter- mine the effect of three different biological process variables: temperature, incubation time, and Kefir grains con- centration on the removal efficiencies of COD, PO_4^{3-} and NO3−. Experimental results revealed that the incubation time factor is positively significant on the percentage of PO_4^{3-} removal. However, the temperature is more significant than Kefir grains concentration and incubation time on the removal of NO3−, and only the interaction effect between the two variables temperature

and Kefir grains concentration is significant. Under the optimum conditions of the grains concentration of 1.02%, temperature at 36.68 °C and incubation time of 5.14 days, about 87% of COD, 37.48% of PO_4^{3-}, and 39.5% of NO3 – were removed after the biological process. The reusability tests of the grains showed that COD removal rate is more than 80% up to two cycles, suggesting that the biological process with Kefir grains could be a promising approach for industrial GCW treatment. After 10 days, a positive effect on barley seedlings growth parameters and chlorophyll a content of was observed with TGCW 50X in comparison with RGCW and control. Future research should focus on testing the effect of TGCW on barley seedlings growing in pots on growth, productivity and antioxidant enzymes activities during prolonged periods (1, 2, and 3months).

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Legend of figures:

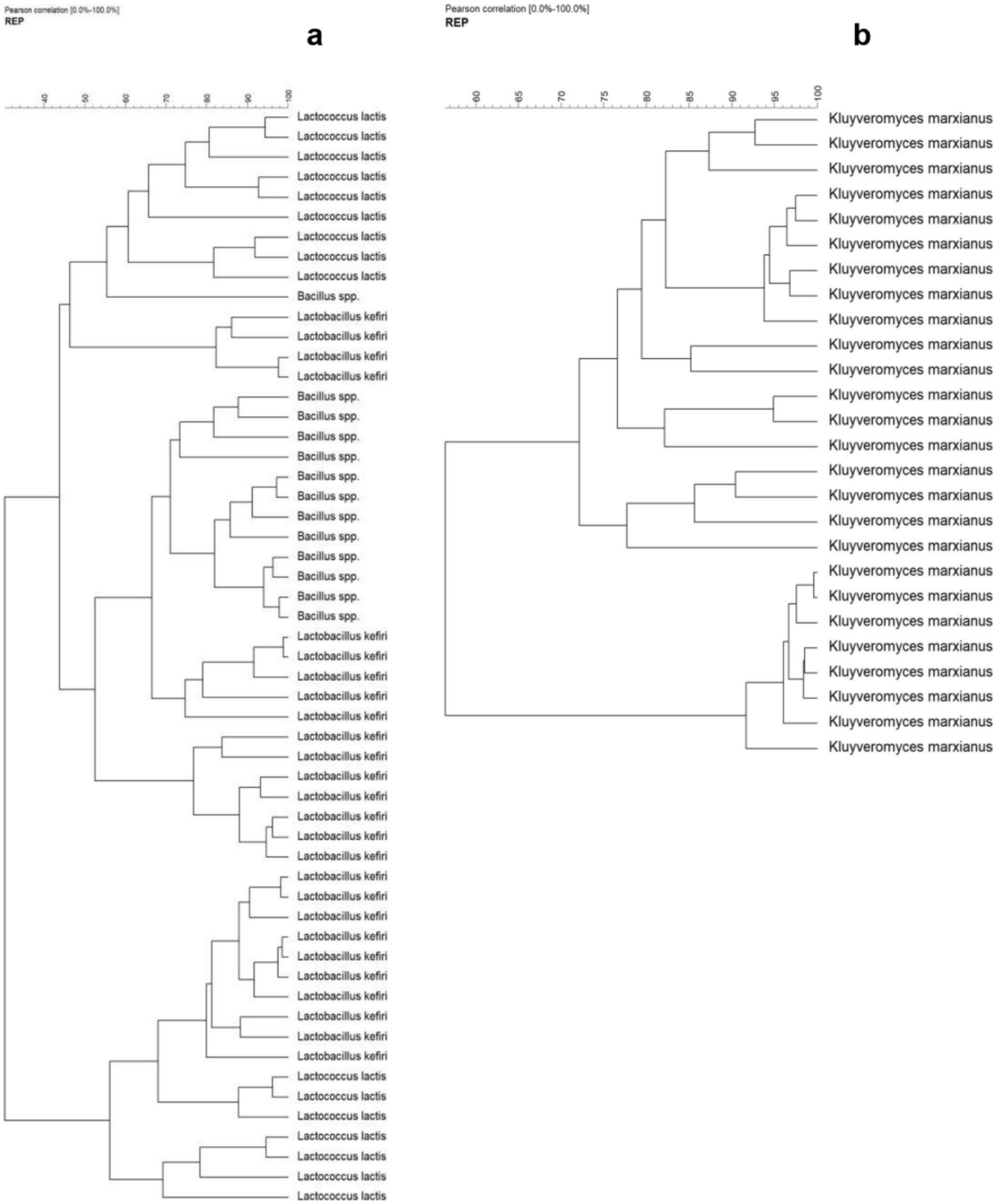
Fig. 1 Dendrograms of bacteria (a)and yeast(b) from Kefir grains obtained by the cluster analysis ofrep-PCR (GTG)5 fingerprints. The dendrogram is based on the Pearson coefficient of similarity with the unweighted pair group method with arithmetic averages clustering algorithm (UPGMA)

Fig. 2 Response surface graphs showing the effect of the interaction between temperature, T (X1), incubation time, t (X2) and Kefir grains concentration, C (%) (X3) on COD (Y1), PO_4^{3-} (Y2), and NO3 − (Y3) removal rates; (av Interaction X1X2,(b)interaction X1X3,and (c) interaction X2X

Fig. 3 Effect of RGCW and TGCW at different dilutions (25, 50, and 100X), on barley germination (a), fresh weight (b), root (c), and shoot length (d)

Fig. 4 Effect of RGCW and TGCW at different dilutions (50 and 100X) on barley growth

Fig. 5 Total chlorophyll (a), chlorophyll a (b), and chlorophyll b (c) contents of barley seedlings soaked with H2O (control), RGCW 50X, and TGCW 50X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Pearson correlation [0.0%-100.0%]
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Figure 1

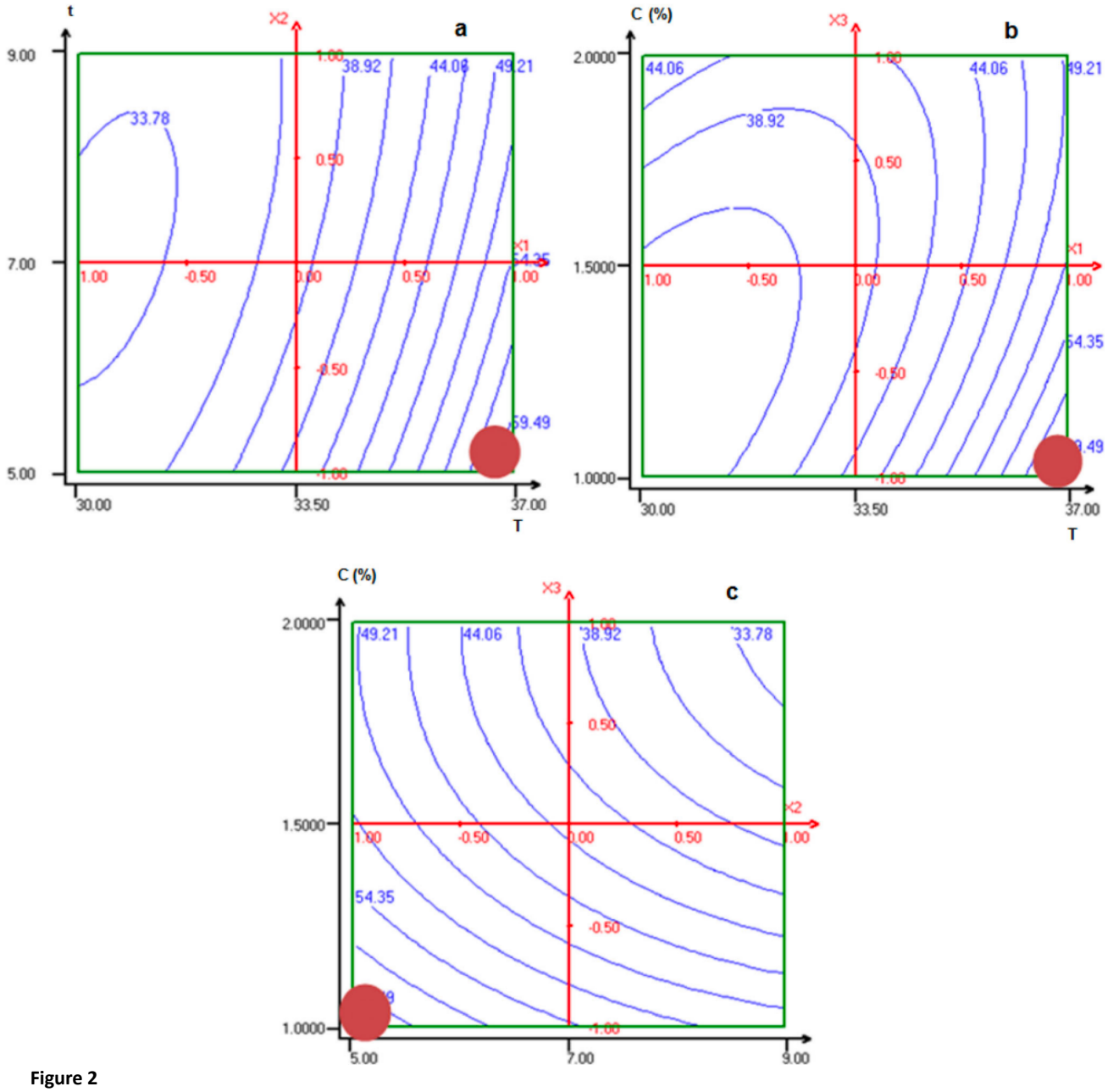
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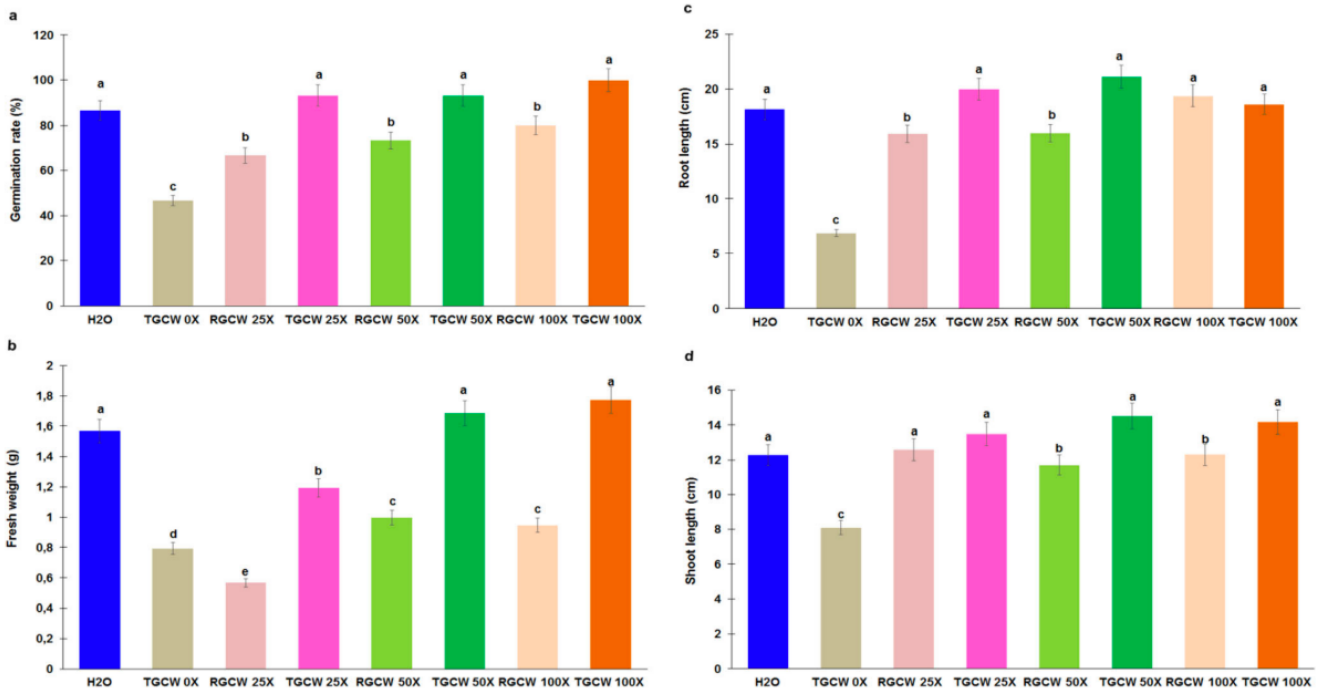
Figure 2



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543 **Figure 3**

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546 **Figure 4**

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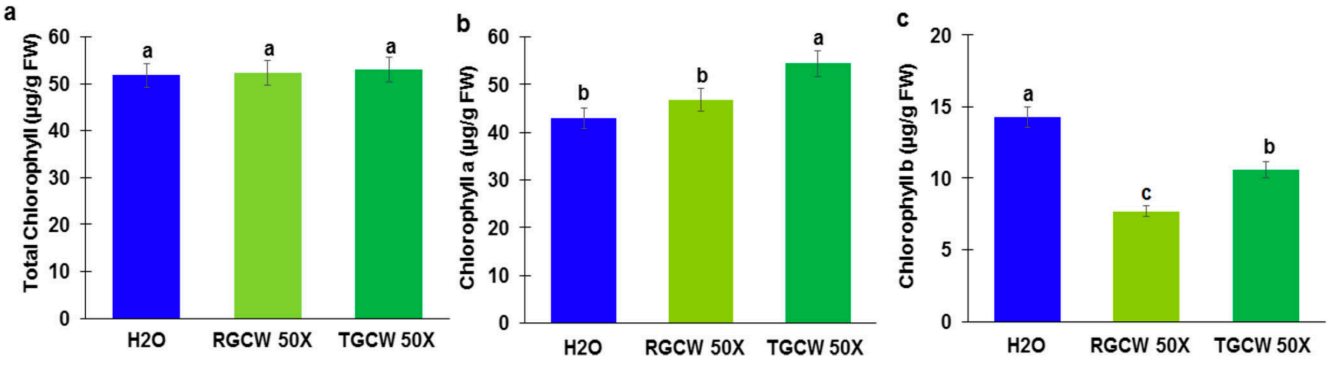

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550 **Figure 5**

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561 **Tables**

562

563 **Table 1** Characteristics of raw cheese whey (CW), white wastewaters (WW) and their mixture (GCW) in ratio 1:1

Parameters	Unit	CW	WW	GCW
pH	–	4.36 ± 0.3	6.09 ± 0.25	4.48 ± 0.5
COD	mg L^{-1}	46,080 ± 24	700 ± 15	25,920 ± 20
BOD_5	mg L^{-1}	19,200 ± 19	–	9800 ± 10
PO_4^{3-}	mg L^{-1}	2.94 ± 0.08	1.88 ± 0.03	2.115 ± 0.05
NO_3^-	mg L^{-1}	3920.95 ± 33	3725.78 ± 25	4309.28 ± 28
Conductivity	mS cm^{-1}	12.47 ± 0.1	0.76 ± 0.05	5.35 ± 0.045
Turbidity	NTU	2144.70 ± 19	2.10 ± 0.047	930 ± 14

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566 **Table 2** Factors and levels of experiment

Levels	Factors		
	X_1 Temperature (°C)	X_2 Incubation time (day)	X_3 Kefir grains concentration (%)
− 1	30	5	1
0	33.5	7	1.5
+ 1	37	9	2

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576 **Table 3** Experimental design matrix and responses (COD, PO_4^{3-} and NO3 − removal rates) during Kefir grains
577 treatment using different concentrations of Kefir grains (1; 1.5 and 2%), at 30; 33.5 and 37 °C during 5; 7 and 9
578 days

Experiment	Independent variables			Responses (Y, removal rates %)					
	X_1 (°C)	X_2 (day)	X_3 (%)	COD		PO_4^{3-}		NO_3^-	
	Temperature	Incubation time	Kefir grains concentration	O*	P*	O	P	O	P
1	30	5	1.5	50	59.09	31.46	31.64	38.39	35.92
2	37	5	1.5	66.66	67.42	38.16	39.90	53.28	52.10
3	30	9	1.5	72.22	71.46	50.63	48.89	31.94	33.121
4	37	9	1.5	61.11	52.02	39.13	38.95	35.40	37.87
5	30	7	1	66.67	67.43	42.01	40.57	36.82	33.32
6	37	7	1	61.11	70.20	41.52	38.52	59.49	54.70
7	30	7	2	72.22	63.13	34.33	37.33	35.06	39.85
8	37	7	2	50	49.24	36.25	37.69	35.89	39.39
9	33.5	5	1	88.88	79.03	35.77	37.03	36.40	42.37
10	33.5	9	1	66.67	66.67	41.52	44.70	34.57	36.89
11	33.5	5	2	55.55	55.55	37.69	34.51	43.33	41.01
12	33.5	9	2	55.05	64.90	44.40	43.14	35.43	29.46
13	33.5	7	1.5	72.22	68.52	30.02	32.60	33.90	32.29
14	33.5	7	1.5	66.67	68.52	38.23	32.60	31.77	32.29
15	33.5	7	1.5	66.67	68.52	29.54	32.6	31.21	32.29

579 O^* observed, P^* Predicted

580

581 **Table 4** Statistical analysis of BBD and significance of the independent variables and their interactions for COD
582 removal (%)

Coefficient	Value	SD	*t* student	Signification (%)
a_0	68.52	6.04	11.34	***
a_1	− 2.78	3.70	− 0.75	49.10
a_2	− 0.76	3.70	− 0.20	84.0
a_3	− 6.31	3.70	− 1.71	14.70
a_{11}	− 5.03	5.44	− 0.92	40.10
a_{22}	− 0.99	5.44	− 0.18	85.60
a_{33}	− 0.99	5.44	− 0.18	85.70
a_{12}	− 6.94	5.23	− 1.33	24.10
a_{13}	− 4.17	5.23	− 0.80	46.60
a_{23}	5.43	5.23	1.04	34.90

583 ***$P < 0.1\%$; *SD* standard deviation

584

585 **Table 5** Statistical analysis of BBD and significance of the independent variables and their interactions for

586 PO_4^{3-}removal (%)

Coefficient	Value	SD	*t* student	Signification (%)
b_0	32.60	2.57	12.67	***
b_1	− 0.42	1.58	− 0.27	79.40
b_2	4.08	1.58	2.59	*
b_3	− 1.02	1.58	− 0.65	55.10
b_{11}	2.97	2.32	1.28	25.7
b_{22}	4.28	2.32	1.85	12.3
b_{33}	2.95	2.32	1.28	25.7
b_{12}	− 4.55	2.23	− 2.04	9.5
b_{13}	0.60	2.23	0.27	79.2
b_{23}	0.242	2.23	0.11	91.5

587 ***$P < 0.1\%$, *$P < 5\%$, *SD* standard deviation

588

589 **Table 6** Statistical analysis ofBBD and significance ofthe independent variables and their interactions for NO3

590 − removal (%)

Coefficient	Value	SD	t-student	Signification (%)
c_0	32.29	0.82	39.41	***
c_1	5.23	0.50	10.42	**
c_2	- 4.26	0.50	− 8.48	*
c_3	− 2.20	0.50	− 4.38	*
c_{11}	5.92	0.74	8.02	*
c_{22}	1.54	0.74	2.08	17.3
c_{33}	3.60	0.74	4.88	*
c_{12}	− 2.86	0.71	− 4.03	5.4
c_{13}	− 5.46	0.71	− 7.69	*
c_{23}	− 1.52	0.71	− 2.14	16.7

591 ***$P < 0.1\%$, **$P < 1\%$, *$P < 5\%$, *SD* standard deviation

592

Table 7 Analysis ofvariance results for COD, PO_4^{3-},and NO3− removal (%)

Response (Y, %)	Source of variance	SS	DF	MS	Ratio	Signification (%)
COD removal	Regression	861.20	9	95.69	0.87	59.5
	Residual	547.19	5	109.44		
	Lack of fit	526.66	3	175.55	17.10	5.5
	Pure error	20.53	2	10.27		
	Total	1408.39	14			
PO_4^{3-} removal	Regression	342.96	9	38.11	1.92	24.40
	Residual	99.32	5	19.86		
	Lack of fit	51.61	3	17.20	0.72	62.50
	Pure error	47.72	2	23.86		
	Total	442.29	14			
NO_3^- removal	Regression	732.58	9	81.40	40.41	0.039***
	Residual	171.35	5	34.27		
	Lack of fit	167.35	3	55.78	27.69	3.51*
	Pure error	4.029	2	2.01		
	Total	903.94	14			

SS: Sum of Squares, DF: Degree of Freedom, MS: Mean Square, *** $P < 0.1$ %, * $P > 5$%

Table 8 Reusability test results of Kefir grains after two cycles for COD, PO_4^{3-},and NO3 − removal from GCW using 1.02% Kefir grains at 36.68 °C during 5.14 days

	Kefir grains treatment		
	Pollutants removal rates (%)		
	COD	PO_4^{3-}	NO_3^-
Native	87 ± 0.5	37.48 ± 0.74	39.5 ± 0.39
Cycle 1	80 ± 0.33	65.3 ± 0.45	80.5 ± 0.28
Cycle 2	82.6 ± 0.20	30.8 ± 0.35	32.3 ± 0.27