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RESEARCH PAPER

Development of a Microbial Consortium for Dairy Wastewater Treatment

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Abstract The wastewater from the dairy industries usually contains high concentrations of contaminants and, since the volume generated is also high, the total contaminant load is very significant. Among the available options for treatment, biological degradation looks like the most promising one. Furthermore, the supplementation of the native microbial populations with external microorganisms with high specific degradation rates (bio-augmentation) has demonstrated to improve the performance of treatment. The main objective of this research was to select a combination of bacteria to improve the aerobic treatment of dairy processing wastewater. For this purpose, eleven fat/protein-degrading microorganisms belonging to the genera *Bacillus*, *Serratia*, *Lactococcus*, *Enterococcus*, *Stenotrophomonas*, *Klebsiella and Escherichia*, were evaluated as potential degrading bacteria using a Plackett-Burman design. Assays were carried out to select the strains that most significantly influenced the degradation of wastewater and biomass yield, in terms of COD removal. A simulated dairy industry effluent was used as culture medium. Four strains were selected as potential members of the microbial consortium: *Lactococcus garvieae*, *Bacillus thuringiensis*, *Escherichia coli* and *Stenotrophomonas* sp. The optimal operation temperature and pH range of the selected consortium were 32°C and 6 ~ 8, respectively. The degradation percentages reached with the selected consortium were 80.67 and 83.44% at 24 and 48 h, respectively. The selected consortium significantly improved the degradation of the dairy wastewater, and the degradation degree achieved by this consortium was higher than by using the strains individually.

Keywords: dairy wastewater, biodegradation, bio-augmentation, Plackett-Burman design

1. Introduction

Wastewater from the dairy industry is highly polluting, considering both the volume generated (2 ~ 6 L of effluent per L of processed milk) and its high organic load [1,2]. It is composed of milk lost during the process, whey, and cleaning water. Furthermore, it presents high content of fats, oils, suspended solids, nutrients, ammonia nitrogen and organic matter [1-3]. This kind of waste commonly exhibits a high chemical and biochemical oxygen demand (COD and BOD, respectively) (1,000 ~ 6,000 mgO_2/L in BOD, with a relation of BOD/COD=0.52 for milk) along with a wide range of pH (4.2 ~ 9.4) and temperature [1,2,4]. Thus, a small dairy, including cheese making facilities, with a daily effluent discharge of 600 m^3, generates a polluting load comparable to that of 36,000 people [3].

Among the available options for treatment, biological degradation is one of the most promising for the removal of organic material from dairy wastewater, since its components are highly biodegradable. They can be effectively treated with biological methods such as activated sludge

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process, aerated lagoons, trickling filters, sequencing batch reactors (SBR), up flow anaerobic sludge blanket (UASB) reactors, anaerobic filters, among others [1,5]. However, the sludge formed, especially during the aerobic biodegradation processes, may lead to serious and costly disposal problems [6]. The presence of fats and long chain-fatty acids formed by the hydrolysis of lipids in dairy wastewater causes retardation in methane production, so the anaerobic treatment of this kind of effluent does not generally proceed in a fast and effective manner. In turn, lipids generally cause less problems in aerobic processes [5,7]. Moreover, the degradation activity of the indigenous microorganisms naturally present may not be sufficient to obtain efficient and reliable treatments [8]. Furthermore, bio-augmentation by adding external microorganisms with hydrolytic enzymes and/or high degradation capacity of specific compound has demonstrated to improve the performance of treatment [9,10].

Most of the inoculums commercially available are generally expensive and are designed for conditions (substrate, temperature, pH, among others) that do not match every particular case. Therefore, the design of an inoculum is of paramount importance to the bio-augmentation of each particular wastewater [8]. A mixture of microorganisms may have a cumulative effect on increasing the biomass activity, growth efficiency, and enzyme production. In addition, mixed cultures serve to overcome feedback regulation and catabolic repression, as the products of one microorganism may act as substrate for the other [11]. An interest in the development of microbial consortia – multiple interacting microbial populations– has recently emerged because consortia can usually perform complex functions that individual populations cannot and consortia can be more robust to environmental fluctuations [12]. The mixed cultures have demonstrated better degradative capacities than single strain. The ability of mixed cultures to survive in a non-sterile environment is important in the biodegradation field [13]. The selected combination of bacteria should also be competitive and thereby persist after inoculation [14]. In industrial applications, the use of mixed cultures is often beneficial to reduce costs and increase the profit of the process, due to their improved degradative capabilities, which enable a collaborative use of different substrates [15].

The development of an optimal mixed culture for wastewater degradation that combines more than five different strains is very laborious and time-consuming because of the large number of experimental requirements [16]. The traditional practice, changing one variable at a time by keeping the others at a constant level, has been found inefficient. In this way, a rapid statistical approach enables us to obtain the strains influencing the degradation process with a reduced number of experiments. The Plackett-Burman (PB) design is highly recommended when more than five factors have to be investigated. This statistical design is very useful for economical determination of the main effects, assuming all interactions are negligible [17,18]. The use of this statistical tool to select an optimal mixed culture is novel and allows the reduction the experimental efforts providing a useful tool for such purposes.

The main objective of this research was to select a combination of bacteria to improve the aerobic treatment of dairy processing wastewater. Eleven fat/protein-degrading microorganisms belonging to the genera *Bacillus*, *Serratia*, *Lactococcus*, *Enterococcus*, *Stenotrophomonas*, *Klebsiella* and *Escherichia*, isolated from different sources, were evaluated as potential degrading bacteria on a simulated dairy effluent. In order to select the variables (strains) that significantly improve the wastewater degradation and biomass yield, a Plackett-Burman design was performed. After the selection of the consortium, its capability to degrade a simulated dairy effluent at different temperatures and pH was analyzed, in order to determine both its optimal operating values and its operation range. Once the optimal variables were defined, the kinetics of the consortium was studied in a bioreactor.

2. Materials and Methods

2.1. Source of microorganisms

Microorganisms were isolated from different agro industrial byproducts and wastes in a previous work by Mazzucotelli *et al*. [19]. Eleven strains were selected according to their hydrolytic capabilities on specific substrates and those that had shown significant hydrolytic capabilities with potential application were identified by the polymerase chain reaction (PCR) methodology. They are shown in Table 1.

2.2. Microorganisms culture maintenance

The isolated cultures were maintained on soft Brain and Heart agar (3.5% w/v of agar- agar) at -18ºC. Subcultures were made every 6 months. The strains were activated in two steps. First, a loop of each culture was inoculated in 10 mL Nutrient Broth (NB) and incubated at 35°C for 24 ~ 48 h; subsequently, 2 mL of active culture were centrifuged at 10,000 rpm for 3 min at 4°C. The obtained precipitate was added to 10 mL of fresh NB and statically incubated at 35°C for 24 ~ 48 h.

2.3. Model wastewater (WW)

The culture medium was a modification of the simulated dairy industry wastewater used by Loperena *et al.*[8]: sterile whole milk powder, 2.08 g/L (La Serenísima, Argentina)

Table 1. Source and phylogenetical identification of the selected strains [19]

Microorganisms source	Strain	Name of closest related species*	Similarity* (%)	Accession number
Dried defatted wheat germ	GIIA4	*Serratia liquefaciens strain M11*	99%	JN596115.1
	GIIA15	*Lactococcus garvieae strain LG-ilsanpaik-gs201105*	97%	JN162117.1
	GIIA20	*Enterococcus faecalis strain HN-N2*	99%	FJ378657.2
Undried deffated wheat germ	GIIA1	*Bacillus pumilus strain IK-MB13-518F*	99%	FJ906741.1
	GIIA2	*Serratia proteamaculans*	99%	EF526505.1
Sunflower meal	GIIA3	*Serratia sp. W2Dec25*	99%	JN106438.1
Defatted soy pellet	GIIA7	*Bacillus thuringiensis strain DW-1T*	99%	EU240956.1
	GIIA9	*Stenotrophomonas sp. DIV102*	99%	FN547780.1
Chesse whey	GIIA16	*Klebsiella sp. strain FDY12*	98%	HE612112.1
	GIIA27	*Enterococcus durans strain PL25*	99%	JN792514.1
Brewer's spent grain	GIIA23	*Escherichia coli strain FDY10*	98%	HE605049.1

*Based on a Blast search of the NCBI database.

was added to a saline solution (NH_4Cl 0.57 g/L, KH_2PO_4 0.21 g/L, K_2HPO_4 0.54 g/L, and Na_2HPO_4 0.67 g/L, in distilled water) previously autoclaved (Initial COD = 3,100 mgO_2/L; fat content = 544 mg fat/L, pH = 6.5).

2.4. Experimental design

A Plackett-Burman design was used to screen the strains (FACTORS or VARIABLES) that significantly influenced COD removal and yield coefficient (RESPONSES). This design showed only the main effect of each variable, and the two-factor interactions (interaction among the different strains were not confounded with the main effects) [20].

Eleven factors (strains) were screened in sixteen combinations with four *dummy* variables. The trials were performed in duplicate and the average of the observation was used as the design response. *Dummy* variables are used to estimate experimental errors in data analysis. Each factor was represented at two levels, high and low (denoted as +1 and -1, respectively). The low level refers to the absence of strain in the inoculum (0%) whereas the high level refers to the presence of the strain in the inoculum. The final inoculum concentration was 5% (v/v) in each Erlenmeyer. The Plackett-Burman experimental design is based on the first order model:

$$Y = \beta_0 + \sum \beta_i X_i \quad (1)$$

where Y is the estimated function for the responses (TD and YB), β_0 is the model intercept, β_i are the regression coefficients, and X_i (X_1 to X_{11}) are the eleven factors (strains). The factors that had a confidence level above 95% were considered the most significant factors that affect the responses [17,18].

First, the effect of each variable was determined as the difference between the average value of the response of the experiments at the high level (+) and the average value of the response of the experiments at the low level (-):
The effect $\mathbf{E(x_i)}$ of each factor was determined by the following equation:

$$E(x_i) = \frac{M_i^+ - M_i^-}{N} \quad (2)$$

where M_i^+ and M_i^- are the responses from the trials with factor x_i at high and low levels, respectively, and N is the number of trial divided by two [17,20].

The experimental error was determined as the average square of the *dummy* effects (Ed):

$$VE = \frac{\sum (Ed)^2}{n} \quad (3)$$

where VE is the variance of the effects and n is the total number of *dummy* variables.

The standard error of an effect is the square root of the variance of an effect and the significant level (p-value) of each effect is determined using the Student's t-test.

2.5. Biodegradation tests

Sixteen 100 mL Erlenmeyer flasks (autoclaved at 121ºC, 15 min) containing 30 mL of sterile model effluent were inoculated with the corresponding combination of strains according to the PB design (final inoculum concentration: 5% (v/v); cell concentration approximately: 5×10^9 c.f.u./mL) (Table 2). The flasks containing the WW and the inoculum were placed in an orbital shaker, and the degradation was carried out at 100 rpm and 32ºC. Samples were taken at 24 and 48 h in order to assess differences in the degradation capabilities of individual strains.

The most important process variables during the

Table 2. Plackett-Burman matrix and percentage of total COD removal (TD) and biomass yield coefficient (YB) results

	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	TD (%)		YB (%)	
Run	GIIA1	GIIA4	GIIA15	GIIA20	GIIA2	GIIA3	GIIA7	GIIA9	GIIA16	GIIA27	GIIA23	D1	D2	D3	D4	24 h	48 h	24 h	48 h
1	1	1	1	1	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	59.88	79.02	26.24	25.45
2	1	1	1	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	1	76.20	72.89	14.66	24.06
3	1	1	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	56.10	51.16	25.96	21.56
4	1	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1	45.38	57.81	30.69	50.39
5	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1	1	56.55	29.96	25.48	23.00
6	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	75.48	73.24	9.11	23.38
7	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	1	62.25	68.00	5.69	26.68
8	1	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1	54.95	59.22	46.23	15.58
9	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1	1	71.81	72.81	23.61	35.22
10	-1	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1	1	1	64.76	79.00	18.65	28.81
11	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1	1	1	-1	75.51	77.18	23.86	24.18
12	1	-1	-1	-1	1	1	1	1	-1	1	-1	1	1	-1	-1	58.13	80.17	33.96	23.69
13	-1	-1	-1	1	1	1	1	-1	1	-1	1	1	-1	-1	1	58.13	72.96	5.00	24.65
14	-1	-1	1	1	1	1	-1	1	-1	1	1	-1	-1	1	-1	66.67	75.11	32.88	12.04
15	-1	1	1	1	1	-1	1	-1	1	1	-1	-1	1	-1	-1	64.64	75.04	28.76	34.17
16	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	≈ 0	≈ 0	0.00	0.00

x_1-x_{11}: variables; x_{12}-x_{15}: dummy variables.

fermentation process are pH and temperature [21]. The operating range and optimum value for TD for the selected consortium with respect to culture pH and incubation temperature were obtained. The experiments were conducted in batch at 24 h, in 100 mL Erlenmeyer flasks containing 30 mL of simulated effluent, final inoculum size 5% (v/v) and at 100 rpm agitation speed.

The effect of pH on effluent degradation was assayed by replacing the saline solution of the simulated effluent by phosphate buffer 0.1 M in a pH range of 6.0 ~ 8.0 at 32°C. Likewise, the effect of temperature on the effluent degradation was assayed by incubating the samples at different temperatures: 15, 25, 32, and 37ºC, at pH = 6.5.

Each experiment was performed in triplicate and the results are presented as the mean value ± sd.

The selected strains were assayed both individually and in mixed culture under the previously detailed conditions, at 24 and 48 h (simulated effluent 30 mL; final inoculum size 5% (v/v); 100 rpm agitation speed; optimum pH and temperature). TD for the selected consortium and for each individual strain was analyzed in triplicate.

Kinetic studies were carried out in a jacketed aerated batch stirred tank bioreactor (New Brunswick) of 1.5 L capacity with 1 L working volume. It was fed with the same model effluent and was operated for 48 h with continuous magnetic shaking at 100 rpm. During the test, samples were taken at regular intervals. The temperature and pH were fixed at their optimal values by a temperature controller and phosphate buffer, respectively.

2.6. Determination of degradation parameters and development of kinetic model

The COD was determined according to Standard Methods [22] using method 5520 (Closed Reflux Method). Since the samples presented three phases: a fatty supernatant, an aqueous middle phase and a solid settled; the COD from different phases was measured independently. The non-soluble or solid COD (NSCOD) was the solid settled at the bottom of the tube, the upper non-polar organic phase COD (OCOD) was the supernatant layer, mainly lipids, while the soluble COD (SCOD) was the dissolved organic load placed on aqueous phase between both. These fractions were separated by centrifugation at 10,000 rpm for 15 min at 4ºC. Since there was no solid COD present in the original wastewater, the solid fraction was considered biomass according to Contreras *et al.* [23]. Finally, the total degradation (TD) as percentage of COD removal (Eq. 1) and the organic phase degradation (FD) (Eq. 2) was calculated at any time t_i as:

$$TD_{t=t_i} = 100 \cdot \frac{(SCOD+OCOD)_{t=0} - (SCOD+OCOD)_{t=t_i}}{(SCOD+OCOD)_{t=0}} \quad (4)$$

$$FD_{t=t_i} = 100 \cdot \frac{OCOD_{t=0} - OCOD_{t=t_i}}{OCOD_{t=0}} \quad (5)$$

The biomass yield coefficient (YB) is defined as the average weight of biomass produced per weight of substrate utilized. Increments of bacterial biomass have a positive effect over the kinetic of the process. However the excessive amount of sludge produced during the aerobic wastewater treatment presents practical disadvantages and generates high cost of stabilization [24]. Therefore, very high values of biomass yield coefficients should be avoided in bio-augmentation strategies.

The YB was calculated as:

$$YB_{t=t_i} = 100 \cdot \frac{NSCOD_{t=t_i} - NSCOD_{t=0}}{(SCOD+OCOD)_{t=0} - (SCOD+OCOD)_{t=t_i}} \quad (6)$$

The kinetics was studied by fitting the bioreactor data into the following kinetic model.

The biomass growth rate (r_g) was modeled as a first order power law with the specific growth rate (μ) as parameter.

$$r_g = \mu.NSCOD \quad (7)$$

where, μ is expressed as in the Monod kinetic model.

$$\mu = \frac{\mu_{max}.SCODB}{K_S + SCODB} \quad (8)$$

where μ_{max} is the maximum specific growth rate, K_S is the Monod constant or half saturation constant and SCODB is the aqueous soluble organic fraction that can be degraded by this process. It is calculated from:

$$SCOD = SCODB + SCODI \quad (9)$$

where SCODI is the fraction of aqueous soluble organic load refractory to the process that remains at the end of biodegradation.

Considering that the SCODB is the only growth substrate, the consumption rate of SCODB must be defined as:

$$r_c = \frac{100}{YB} r_g = \frac{100}{YB} \mu.NSCOD \quad (10)$$

where YB is the biomass yield coefficient.

Finally the proposed model assumes that the fats hydrolysis rate (r_H) follow the first order power law.

$$r_H = k_H.OCOD \quad (11)$$

where k_H is the first order constant of hydrolysis.

The fat hydrolysis products (glycerol and fatty acids) are easy to degrade and, therefore, they become SCODB. Therefore a hydrolysis yield coefficient (Y_H) was defined as the mass of fat hydrolysis products divided by the mass of substrate (fat) to relate OCOD consumption and SCOD generation. In this work Y_H was assumed to be equal to one.

Later, after solving the mass balance for different organic fractions using the equations (7) to (11), this ordinary differential equation (ODE) system was used for results:

$$\frac{dOCOD}{dt} = -r_H = -k_H.OCOD$$

$$\frac{dSCODB}{dt} = r_H - \frac{100}{YB} r_g = k_H.OCOD - \frac{100}{YB} \frac{\mu_{max}.SCODB}{K_S + SCODB}.NSCOD$$

$$\frac{dNSCOD}{dt} = r_g = \frac{\mu_{max}.SCODB}{K_S + SCODB}.NSCOD \quad (12)$$

The YB was estimated with Eq. 6, at final time. The parameters k_H, μ_{max} and K_S were obtained by fitting to experimental data with the minimum square method on Origin 8.0 ® (OriginLab Corporation). After of parameters estimation the system of equation 12 was solved with a four order Rounge Kutta routine to obtain the organic fraction profiles (MathCad 14®, Mathsoft Corporation).

2.7. Statistical analysis

After conducting experimental trials according to the PB design, COD removal (TD) and yield (YB) results were evaluated using the following statistical parameters: β coefficients (Eq. 1), sum of squares (SS), percentage contribution to the SS, *t*-value and *p*-value. Factors were screened at a confidence level of 95% based on their effects. If a factor showed significance at or above a 95% confidence level and its main effect was positive, a higher concentration would be required for further optimization studies. If the effect was negative, the criterion was to remove the strain from the consortium. Data was analyzed using the REG procedure of SAS (version 8.0, SAS Institute, USA).

On assays different to PB design, the one-way analysis of variance (ANOVA) was used to test for statistical significance among treatments. Each experiment was performed in triplicate and the results are presented as the mean value ± sd. The means were then compared by Tukey's test. Statistical significance was accepted at $p < 0.05$. Data was analyzed using R software (2.14.0 software version).

3. Results and Discussion

Table 2 shows the PB matrix, percentage of COD removal (TD) and biomass yield coefficient (YB) data. Degradation at 24 h shows the influence of faster strains in the degradation process whereas the degradation at 48 h shows the influence of different strains to provide a good final quality of the effluent. While the TD at 24 h allows the evaluation of the strains that reach a high degradation level in a short period of time, the TD after 48 h provides the additional information of the maximum possible degradation

Table 3. Statistical parameters obtained from total COD removal (TD) and biomass yield coefficient (YB) at 24 h

Coded variable	Strain	Effect	SS	Contribution to total SS (%)	*t*-value	*p*-value
TD						
X1	GIIA1	2.875	2.135	0.09	0.834	0.451
X2	GIIA4	5.375	26.083	1.14	1.559	0.194
X3	GIIA15	7.375	92.448	4.04	2.139	0.099
X4	GIIA20	6.125	43.982	1.92	1.776	0.150
X5	GIIA2	-1.125	0.050	0.00	-0.326	0.761
X6	GIIA3	-0.875	0.018	0.00	-0.254	0.812
X7	GIIA7	10.875	437.086	19.09	3.154	0.034
X8	GIIA9	11.375	523.185	22.85	3.299	0.030
X9	GIIA16	2.625	1.484	0.06	0.761	0.489
X10	GIIA27	3.625	5.396	0.24	1.051	0.352
X11	GIIA23	13.875	1158.196	50.57	4.024	0.016
YB						
X1	GIIA1	8.765	184.454	17.59	1.523	0.202
X2	GIIA4	5.372	26.032	2.48	0.933	0.403
X3	GIIA 15	-2.177	0.702	0.07	-0.378	0.724
X4	GIIA20	0.412	0.001	0.00	0.072	0.946
X5	GIIA2	10.567	389.692	37.15	1.836	0.140
X6	GIIA3	2.222	0.762	0.07	0.386	0.719
X7	GIIA7	-3.858	6.920	0.66	-0.670	0.539
X8	GIIA9	5.989	40.204	3.83	1.041	0.357
X9	GIIA16	6.912	71.330	6.80	1.201	0.296
X10	GIIA27	10.088	323.622	30.85	1.753	0.155
X11	GIIA23	-3.589	5.182	0.49	-0.624	0.567

over longer periods of time, since no more COD removal was observed after this time at any assay.

It is important to evaluate these different capacities, since some strains can degrade slowly but to a greater extent over longer periods of time, while other strains exhibiting high initial degradation do not reach low final values of COD. The use of mixed cultures allows to faster strains to reduce the duration of treatment or the reactor volume of wastewater treatment while the best degraders can provide a good quality effluent without the need of post treatment.

Table 3 presents the statistical parameters (the effect, sum of squares (SS), contribution to SS, *t*-value and *p*-value) obtained from TD and YB at 24 h.

When the *p*-values obtained for each factor at 24 h were analyzed, it was found that the strains GIIA7, GIIA9 and GIIA23 (code variables X7, X8 and X11, respectively), resulted significant ($p < 0.05$) for the COD degradation (with *p*-values of 0.034, 0.030 and 0.016). The other strains contributed less positively to TD, except for GIIA2 and GIIA3 (code variables X5 and X6) that affected the COD reduction negatively at least in the first 24 h. It is also useful to consider the contribution of each factor to the total sum of squares (SS) of COD reduction. As can be observed in Table 3, the major contributions to the SS are mainly explained by the significant factors on COD removal: the strain GIIA23 contributes with 50.57%; GIIA9 with 22.85%; and GIIA7 with 19.09%, giving the three significant strains a total contribution of 92.51%.

Regarding YB, even though no strain was found to be statistically significant ($p < 0.05$), the analysis of the contribution to the SS allows for the distinction between strains with major contributions on the parameter analyzed. Table 3 shows that the strains that contributed the most to the SS were GIIA2 with a partial SS of 37.15%, GIIA27 with 30.85% and GIIA1 with 17.59%; giving the three strains a total contribution of 85.59%.

The strains GIIA7, GIIA9 and GIIA23 (which correspond to *B. thuringiensis, Stenotrophomonas* sp. and *E. coli*) resulted significant for COD degradation for the first 24 h. On the other hand, these strains were not the ones that contributed the most for YB. Thus, they were preliminarily selected as potential members of the microbial consortium.

Table 4 presents the statistical parameters (the effect, sum of squares (SS), contribution to SS, *t*-value and *p*-value) obtained from TD and YB at 48 h. At this time, it was found that the strains GIIA7, GIIA15, GIIA16 and GIIA23 (code variables X7, X3, X9 and X11, respectively) resulted significant ($p < 0.05$) for TD (with *p*-values of 0.025, 0.026, 0.036, and 0.040). The strain GIIA9 (variable code X8), with a *p*-value at the limit of significance (0.051) and 9.52% total sum of squares contribution, proved also to be significant for the reduction of COD at 24 h. The remaining strains contributed less positively to TD. Based

Table 4. Statistical parameters obtained from total COD removal (TD) and biomass yield coefficient (YB) at 48 h

Coded variable	Strain	Effect	SS	Contribution to total SS (%)	*t*-value	*p*-value
TD						
X1	GIIA1	9.125	216.665	1.66	1.787	0.148
X2	GIIA4	0.625	0.005	0.00	0.122	0.908
X3	GIIA15	17.625	3015.550	23.07	3.451	0.026
X4	GIIA20	4.875	17.650	0.14	0.955	0.394
X5	GIIA2	3.375	4.055	0.03	0.661	0.545
X6	GIIA3	12.875	858.695	6.57	2.521	0.065
X7	GIIA7	17.875	3190.320	24.41	3.500	0.025
X8	GIIA9	14.125	1243.953	9.52	2.766	0.051
x9	GIIA16	15.875	1984.746	15.18	3.109	0.036
X10	GIIA27	12.625	793.918	6.07	2.472	0.069
X11	GIIA23	15.375	1746.266	13.36	3.011	0.040
YB						
X1	GIIA1	5.723	33.533	6.68	0.781	0.478
X2	GIIA4	-0.439	0.001	0.00	-0.060	0.955
X3	GIIA 15	7.139	81.174	16.17	0.975	0.385
X4	GIIA20	0.758	0.010	0.00	0.104	0.923
X5	GIIA2	2.787	1.886	0.38	0.380	0.723
X6	GIIA3	3.052	2.711	0.54	0.417	0.698
X7	GIIA7	2.019	0.519	0.10	0.276	0.797
X8	GIIA9	0.006	0.000	0.00	0.001	0.999
x9	GIIA16	10.504	380.472	75.81	1.434	0.225
X10	GIIA27	0.330	0.000	0.00	0.045	0.966
X11	GIIA23	-2.658	1.559	0.31	-0.363	0.735

on SS, the strains ($p < 0.05$) GIIA7, GIIA15, GIIA16 and GIIA23 were the major contributors to COD reduction, with partials SS of 24.41, 23.07, 15.18 and 13.36%, respectively (Table 4). The four significant strains summed a total contribution of 76.02%. Considering the strain GIIA9 (with a partial SS of 9.52%), the total contribution achieved was 85.54%.

Regarding YB, although none of the strains had significant impact ($p < 0.05$) on this parameter, two of the strains contributed significantly to the SS: GIIA16 with a partial SS of 75.81% and GIIA15 with a partial SS of 16.17%; providing both strains a total contribution of 92.08%.

Considering that the strains GIIA15, GIIA7, GIIA9, GIIA23 and GIIA16 (which correspond to *L. garvieae*, *B. thuringiensis*, *Stenotrophomonas* sp., *E. coli* and *Klebsiella* sp.) resulted significant for COD removal at 48 h, and taking into account that the *Klebsiella* sp. strain contributed significantly to the SS of the YB (75.81% at 48 h), only the first four were considered in the preliminary selection for the microbial consortium.

From the results obtained in the assays at 24 and 48 h, the strains selected as potential members of the microbial consortium were: *L. garvieae*, *B. thuringiensis*, *E. coli* and *Stenotrophomonas* sp.

Fig. 1 shows the TD by the selected consortium, at different pH values (A) and temperatures (B). No significant differences were observed on TD at different pHs over the range tested at 32°C ($p = 0.617$). The average COD degradation values obtained was 80.76%. These results indicate that the selected consortium could operate within the range tested with the same performance. Therefore, this consortium presents robustness to environmental fluctuations. This is of paramount importance when an inlet fluctuation takes place due to variations in discharges of the process lines.

Regarding the assay at different temperatures, significant differences in TD between samples were observed ($p < 0.05$). The higher degradation value was observed at 32°C (80% COD removal). Samples incubated at 25 and 37°C showed a significantly lower degradation (around 60% COD removal), but without significant differences between them. Therefore, there is a 25% reduction in TD in a range of ± 6ºC compared to the optimum. On the other hand, the incubation at 15°C reached the lowest degradation value (33%). Then, the parameters selected for effluent degradation by the mixed culture were 32ºC and the original pH of the simulated effluent (pH 6.5).

In these conditions, the selected strains were assayed both individually and as in mixed culture. Fig. 2 shows the results of TD and FD for each strain alone and for the selected microbial consortium. Higher values of total degradation are achieved by the selected consortium both at 24 and 48 h (80.67 and 83.44%, respectively). *Stenotro-*

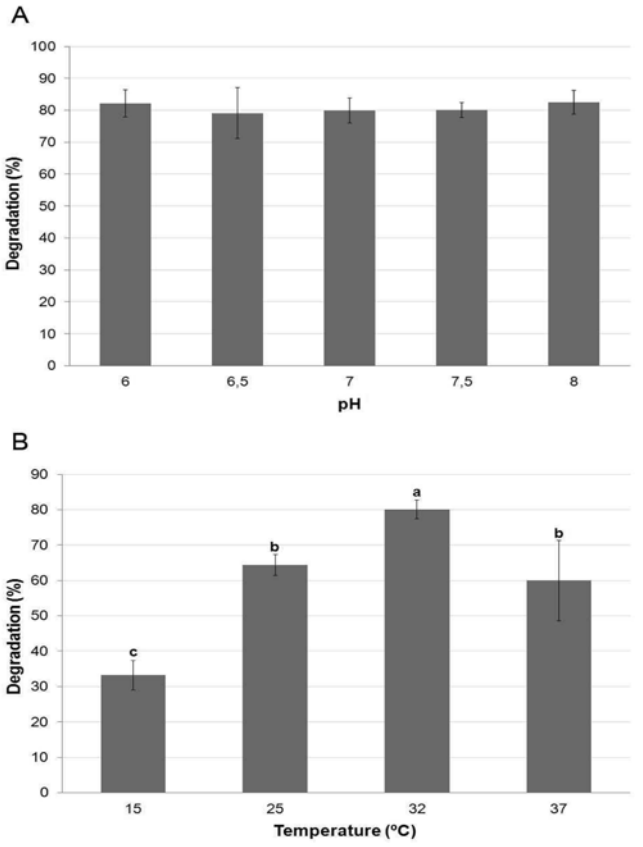

Fig. 1. Total degradation percentage (TD) of a model effluent by the selected consortium at 24 h for different pHs at 32°C (A) and temperatures at pH = 6.5 (B). Values represent means of three independent experiments and error bars indicate standard deviations. For each incubation temperature, the same letter above bars indicates the absence of significant differences (Tukey's test, $p \leq 0.05$).

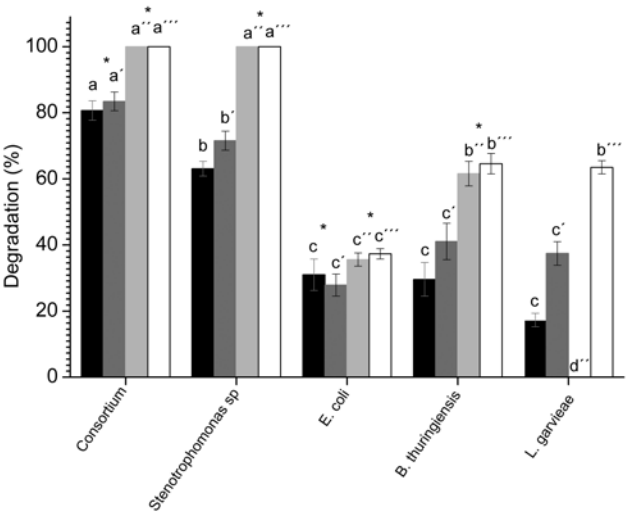

Fig. 2. Total degradation percentage (TD) at 24 h (■) and 48 h (■) and organic phase degradation (FD) at 24 h (■) and 48 h (□) for each selected strain alone and for the microbial consortium (pH = 6.5, T = 32°C). Different letters in the same time indicate a significant difference between samples. The asterisk (*) indicates no significant difference between 24 and 48 h for the sample (Tukey's test, $p \leq 0.05$).

phomonas sp. reached high degradation values at both times (63.05 and 71.57% at 24 and 48 h, respectively), significantly different from the other individual strains, but significantly lower than those achieved by the microbial consortium. The total degradation reached by both *B. thuringiensis*, *L. garvieae* and *E. coli* showed no significant differences at both times. On the other hand, both the *Stenotrophomonas* sp. strain and the selected consortium has been achieved the depletion of the upper organic phase at 24 h. The other three strains individually produced significantly less degradation of the organic phase. It is also important to note that the FD did not differ significantly at 24 and 48 h in the presence of *B. thuringiensis* and *E. coli*, whereas in the presence of *L. garvieae* the upper organic phase remained intact during the first 24 h, but at 48 h reached similar levels to those achieved by *B. thuringiensis*. The four selected strains have been reported in previous researches as having multienzyme capacities [19,25-27]. Particularly, Mazzucotelli *et al.* [19] highlighted the amylolytic, proteolytic, caseinolytic, lipolytic and cellulolytic activity of *B. thuringiensis* and *Stenotrophomonas* sp. strains. Furthermore, the analysis of the hydrolytic capacities and degradation degree achieved by individual strains and the consortium, shows that the use of mixed populations can perform functions which are difficult or even impossible for individual strains, due to the complementary effects that exist between the metabolism of the strains involved [8,12,16,28,29].

Although 100% COD removal has been reached on thermophilic conditions at 65°C [30], the energetic requirements of this technology make it unpractical. The TD values higher than 80% found for consortium fall near the top of the range of values reported on literature for dairy wastewater treated by mixed consortia [8,11,31]. They are even higher than those obtained for anaerobic-aerobic treatment schemes [30]. Furthermore, TD for this consortium is higher than that obtained with commercial inoculums, which reached values of 63% for 48 h [8]. The TD achieved by the consortium at 24 h was between 2.7 and 6 fold higher than *B. thuringiensis*, *L. garvieae* and *E. coli*. It is also observed that for the consortium there are no significant differences between the 24 and 48 h.

As mentioned above, at 24 h both the consortium and the *Stenotrophomonas* sp. strain alone have achieved the complete disappearance of the upper phase, mainly composed by lipids. The high degradation capacity of *Stenotrophomonas* sp. is directly associated with its various enzymatic capabilities [19]. Another strain that showed a high degradation degree of the organic phase was

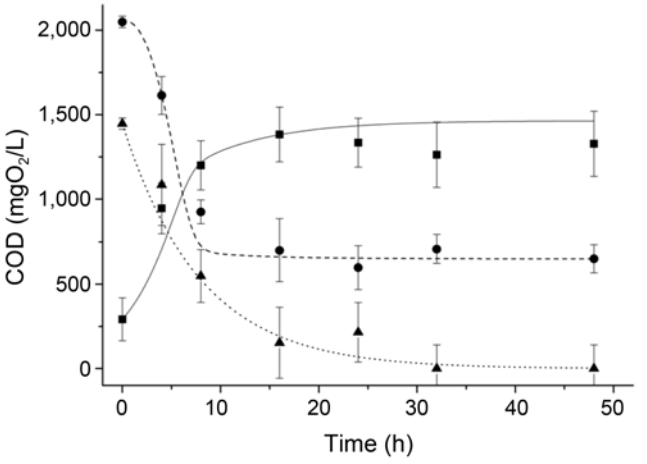

Fig. 3. Profiles of different organic fractions: NSCOD (■), SCOD (●), and OCOD (▲). The lines represent the prediction of the model. NSCOD (solid), SCOD (dashed), and OCOD (pointed).

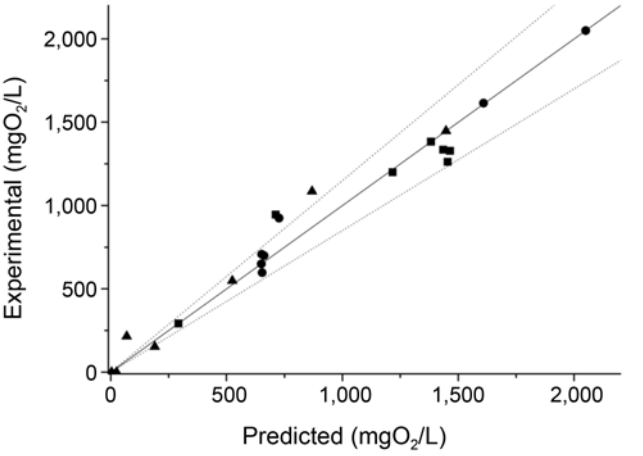

Fig. 4. Parity plot of proposed model, experimental *vs* theoretical values: NSCOD (■), SCOD (●), and OCOD (▲). The dashed lines represent 15% error.

B. thuringiensis, reaching mean degradation values of 63%. When analyzing the degradation performed by *L. garvieae* it can be noticed that in the first 24 h the organic phase remains intact without degradation, and at 48 h it reaches values of 64%. *L. garvieae* probably has preference for other biomolecules as primary energy source, such as the lactose present in the soluble phase, and consumption of the organic phase begins after carbohydrates depletion.

The behavior of different COD organic fractions versus time in the bioreactor is showed in Fig. 3 with dots. It is important to note that at 8 h, the consortium achieved a 50% of TD, while at 16 h the degradation values obtained were very close to the maximum (75.61%) which implies an advantage from the point of view of time process or reactor volume. In addition, the consumption rate of SCOD was higher than the hydrolysis rate. It is important to note that the consumption of the OCOD phase was the limiting stage of the process, according with previous reports [5,32]. For this reason, the efforts to improve the global degradation rate should be focused on this stage.

Fig. 3 shows with lines the prediction of the model depicted by the system of equations (Eq. 12). The values obtained for the parameters were: YB = 41.2%; k_H = 0.1265 1/h; μ_{max}= 0.301 1/h and K_S = 450 mg O_2/L. The value of μ_{max} found is higher than previously reported [33,34]. This clearly indicates that the consortium selected can degrade dairy WW faster. Fig. 4 shows in a parity plot how the values predicted by the model adjust properly to experimental data. The dashed lines indicate a 15% error. The ANOVA test showed that the population means are not significantly different in all the cases. This model together with an appropriate characterization will allow its application for the design of a full-scale aerobic reactor and its optimization. In addition, this work provides a useful tool for industrial application since a complex degradation of dairy wastewater can be modeled based on an easy to measure, global parameter, such as COD.

4. Conclusion

The Plackett-Burman design has demonstrated to be a useful tool to select a bacteria combination to improve the aerobic treatment of dairy wastewater. The values of TD achieved by the selected consortium, both at 24 and at 48 h at optimal pH and temperature (6.5 and 32°C, respectively), were greater than those achieved for any combination of strain tested with a Plackett-Burman assay. The selected consortium presented the highest level of conversion, at both 24 and 48 h. Thus, the consortium has demonstrated its suitability to be applied for technological purposes in dairy effluent degradation considering operating costs, volume of bioreactor and the degree of degradation achieved. Finally, the developed model has demonstrated its capability to predict the behavior of the system and can be used for further research work or a more complex reactor design.

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