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Mixed Culture Optimization for Marigold Flower Ensilage via Experimental Design and Response Surface Methodology

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Endogenous microorganisms isolated from the marigold flower (*Tagetes erecta*) were studied to understand the events taking place during its ensilage. Studies of the cellulase enzymatic activity and the ensilage process were undertaken. In both studies, the use of approximate second-order models and multiple lineal regression, within the context of an experimental mixture design using the response surface methodology as optimization strategy, determined that the microorganisms *Flavobacterium IIb*, *Acinetobacter anitratus*, and *Rhizopus nigricans* are the most significant in marigold flower ensilage and exhibit high cellulase activity. A mixed culture comprised of 9.8% *Flavobacterium IIb*, 41% *A. anitratus*, and 49.2% *R. nigricans* used during ensilage resulted in an increased yield of total xanthophylls extracted of 24.94 g/kg of dry weight compared with 12.92 for the uninoculated control ensilage.

KEYWORDS: Marigold flower; ensilage; starter inoculum; mixed culture

INTRODUCTION

The oleoresin obtained from marigold flower (*Tagetes erecta*) is used commercially as an additive to poultry feed to improve the birds' nutrition and the pigmentation of their skin and egg yolks. A bright yellow color is often associated with good health and premium quality by the consumer (*1*, *2*). Carotenoids, in particular lutein, the main oleoresin component, has demonstrated properties as an anticarcinogen, as an agent to facilitate ligament repair, as an aid in enzyme transport, and as a preventative agent for age-related macular degeneration (AMD), a leading cause of visual problems in the elderly (*3*, *4*). These properties have sparked renewed interest in the development of alternative production routes for carotenoid pigments such as chemical synthesis (*5*) and fermentation technology (*6*−*8*). Complementary efforts have been directed toward increasing the extraction yield from marigold flower flour using supercritical fluid extraction (*9*) and enzymatic preparations (*10*). In broad terms, the commercial extraction of oleoresin from marigold flower consists of five stages: ensilage, pressing, drying, hexane extraction, and saponification. Ensilage efficiency has been identified as the main factor hindering industrial recovery of xanthophylls via solvent extraction (Alcosa S. A., Celaya, Gto. México). In this stage, the microorganisms associated with the marigold flower degrade the cellulose and hemicellulose present in the cell walls of the flower petals. This degradation increases the mass exchange between solvent and solid in the extraction process.

In general, in the fermentation processes two types of microorganism are used: (i) endogenous microorganisms isolated from natural sources and (ii) pure culture microorganisms obtained from collections. In both cases, the microorganisms can be used alone or as part of a mixed culture in the preparation of starter inoculum for the fermentation. Mixed cultures are used in traditional fermented foods, often determining their texture and flavor, and in the treatment of hazardous and nonhazardous wastes (*11*, *12*). In these applications, however, the interactions among the components of the mixed culture are poorly understood. Several simple mathematical models have been proposed to predict the activity of mixed cultures; however, these predictions are inaccurate for mixtures of greater than two microorganisms (*13*). An alternative strategy to characterize mixed cultures relies on preparing and testing mixed cultures of variable composition. The obvious drawback of this strategy is the time and resources required to obtain data (*14*, *15*). A statistically based experimental design can be used for optimization purposes, allowing a search over several variables with a reduced number of experiments. This strategy relies on several standard statistical tools globally known as response surface methodology (*16*, *17*). Response surface methodology can be also used for mixture designs. In a mixture design, the factors are components or ingredients of a mixture, and consequently, their levels are not independent: if x_1, x_2, ..., x_p denote the proportions of p components of a mixture,

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then the levels are fractions and their sum should be equal to 1 [$(0 \leq x_i \leq 1)$ and $(x_1 + x_2 + ... + x_p = 1)$] (*16*). Mixture design based on statistical experimental design has been successfully used to evaluate the influence of sugars and acids in mango flavor (*18*), to study the effect of nutrients in soybean growth and its subsequent assimilation by herbivores (*19*), and to study the efficacy of pesticide applications (*20*).

The main objective of this study is to characterize the effect of endogenous microorganisms on marigold flower ensilage efficiency and the total xanthophyll extraction yield. We address the optimization of the culture used in the ensilage as an alternative to improve the efficiency of the overall extraction. The illustration presented here includes a study of the enzymatic activity of the microorganisms in a model cellulose solution. The results on these assays allow us to gain a partial understanding of the events taking place during the ensilage of marigold flowers and explain the mixed culture composition which gives optimal extraction yield. We also demonstrate the feasibility of an experimental mixture design combined with surface response methodology to quantify the relative importance of a microorganism within a mixed culture, leading to the optimization of the starter culture inoculum.

MATERIALS AND METHODS

Fresh Material. Fresh marigold flowers (*T. erecta*) were supplied by Industrias Alcosa, S. A. de C. V. Guanajuato, México. A single batch was used for all experiments. The flowers were separated from the receptacles and the petals were mixed until a homogeneous sample was obtained (visual inspection).

Microorganism. Microorganisms associated as normal flora of the marigold flower [*Flavobacterium IIb* (C_1), *Acinetobacter anitratus* (C_2)-, *Enterobacter intermedius* (C_3), *E. aerogenes* (C_4), *E. agglomerans* (C_5), *Rhizopus nigricans* (H_1) *and Geotrichum candidum* (H_2)] were previously isolated and identified following the techniques described in refs *21* and *22*. To evaluate the effect of the microorganisms, by themselves or within a mixed culture, on the ensilage of fresh marigold petals and their enzymatic activity via essays on a carboxymethyl cellulose (CMC − high viscosity; Sigma Chemical Co., St. Louis, MO) solution, microorganisms from the same pure culture were used for all treatments.

Culture Preparation. The microorganisms were cultured on nutrient and potato dextrose (PDA) agars (Sigma Chemical Co., St. Louis, MO) slants at 28 °C for 24 h. Biomass taken from the slants was transferred to 250 mL Erlenmeyer flasks containing 100 mL of potato dextrose or nutrient (for fungi and bacteria respectively) broth (Sigma Chemical Co., St. Louis, MO), incubated on a rotary shaker at 28 °C and 175 rpm (Forma Scientific, model 4520) for 30 h. Samples were taken every 2 h for kinetic growth and enzymatic activity studies. The kinetics studies allowed us to determine the time (microorganism's growth phase) at which the largest amount of cellulase is expressed by a particular microorganism.

Microorganism Growth. For determining cellular dry weight (cdw), a known fermentation broth volume was filtered using Millipore membranes (0.45 μm pore size). The membranes and cell pellets were then dried at 90 °C for 12−16 h, until a constant weight was obtained.

Enzymatic Activity. In preliminary experiments, we found that the cellulase is expressed and excreted into the growth medium by each microorganism. These preliminary experiments consisted of electrophoresis over standard SDS−polyacrylamide gels with concentrations in the range 3−20% (*23*). Gels exposed to supernatant without microorganisms, supernatant with lysed microorganisms, and cellulase from *Aspergillus niger* (Merck KGaA, Darmstadt, Germany) were examined. Our results showed that the gels exhibited the same bands with similar intensity at the same locations, indicating that the cellulase is expressed and excreted by the microorganisms under study.

For enzymatic activity studies, the biomass obtained from the Erlenmeyer flask propagation was centrifuged at 6000 rpm (Hermle, model Z383). The supernatant obtained was used as raw cellulase extract (ϵ). A 20 mL volume of raw extract was added to 100 mL of a mixture of carboxymethyl cellulose of 2 g/L that had a viscosity (η) of 4500 centipoises (cp) (Schoot Visco Easy-L). The solutions obtained were kept on a rotary shaker at 28 °C at 175 rpm (Forma Scientific, model 4520) for 24 h. The enzymatic activity was measured indirectly as a function of viscosity (η) reduction.

Mathematical Methods. We consider here a batch fermentation model in which growth is described by the logistic model (*24*):

$$\frac{dN}{dt} = \mu N\left(1 - \frac{N}{N_m}\right) \tag{1}$$

and product formation is described by Leudeking-Piret kinetics (*25*):

$$\frac{dE}{dt} = \alpha\left(\frac{dN}{dt}\right) + \beta N \tag{2}$$

That considers the product formation (enzyme) as a cell mass function. By solving the above equations (with $N = N_0$ and $E = E_0$ at $t = 0$), the expression that models the enzyme production is

$$E - E_0 = \beta\left[N_m t + \frac{N_m}{\mu}\ln\left(\frac{N_0}{N_m} - \left(\frac{N_0}{N_m} - 1\right)l^{-\mu t}\right)\right] + \alpha(N - N_0) \tag{3}$$

where

$$N = \frac{N_0}{\frac{N_0}{N_m} - \left(\frac{N_0}{N_m} - 1\right)l^{-\mu t}} \tag{4}$$

The parameters α and β were calculated from experimental data, using nonlinear optimization via least squares and Levenberg−Marquardt methods, where E is the enzyme concentration, N is the biomass, N_m is the maximum biomass produced, μ is a rate-specific growth constant, and α and β are coefficients that determine whether metabolite production is associated ($\beta = 0$) or not ($\alpha = 0$) with growth. From the experimental data, the enzyme concentration (E) was evaluated as ($V_o - V/V_o - V_f$), where V_o is the initial viscosity, V is the viscosity at a given time, and V_f is the final viscosity.

Ensilage Studies. To evaluate the effect on marigold flower ensilage of a mixed culture or a single microorganism, we used sterile (15 min, 121 °C in a Brinkmann sterilizer model 2540E) fresh marigold flower petals. The petals were placed in Petri dishes, blended with 10 mL of starter inoculum (its composition dictated by the experimental design) and sealed to keep anaerobic conditions. Sterile flower petals were placed in Petri dishes without inoculum as controls. Static samples were incubated over 7 days at 28 °C. The ensiled products were dehydrated in a vacuum oven (Shel lab. model 1430) to 10% (± 1%) moisture content. Dehydrated samples were milled (0.5-mm sieve) using a Brinkmann mill (Brinkmann, Wesbury, NY). The flour obtained was analyzed by AOAC method 970.64 to determine the total xanthophylls concentration (*26*).

Experimental Strategy. During the ensilage studies, two experimental designs were used. First, we applied a *screening* design. Every possible combination of microorganisms was studied using a statistical framework. The results were expressed via a least squares model and used to predict which microorganisms have significant activity in the ensilage process. Second, we devised a *centroid simplex* design, which includes additional runs to account for nonadjustable data, allowing us to estimate the prediction error of the statistical model and find the optimum mixture of microorganisms for the ensilage and enzymatic activity studies (*16*).

Statistical Model. Higher-order terms are frequently necessary in the mixture model because of the constraint $\sum x_i = 1$. Furthermore, complex phenomena that require descriptions encompassing large experimental regions often require elaborate models. However, the terms in these models have relatively simple interpretations. The linear terms describe the expected response for pure cultures ($x_i = 1$ and $x_j = 0$, $j \neq i$), as well as the linear blending portion. When there is curvature arising from nonlinear mixing between component pairs (quadratic terms), the terms represent either synergistic or antagonistic effects

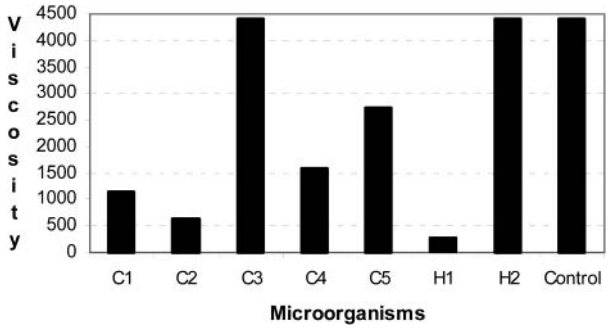

Figure 1. Reduction in viscosity from 4500 cp by raw cellulose enzyme extract (ϵ) in a CMC solution.

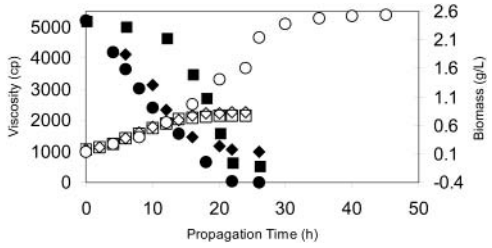

Figure 2. Kinetic growth and cellulase synthesis in batch culture. The open markers indicate the cell concentration in dry weight (right axis) for *Flavobacterium IIb* (◇), *A. anitratus* (□), and *R. nigricans* (○). The filled markers indicate the viscosity change associate with expression of cellulase (left axis) by *Flavobacterium IIb* (♦), *A. anitratus* (■), and *R. nigricans* (●).

dictated by their signs (*16*). The experimental results described below were performed in duplicate, and the reported values represent the mean values of these duplicated experiments.

RESULTS AND DISCUSSION

A qualitative analysis of the change in viscosity observed in the CMC solutions after addition of raw enzyme extract (ϵ) showed that only the bacteria *Flavobacterium IIb* (C_1), and *A. anitratus* (C_2), and the fungus *R. nigricans* (H_1) exhibited significant cellulase enzymatic activity (**Figure 1**). An analysis of the dependence of the enzyme concentration on growth rate revealed that the maximum enzymatic activity occurred at 16, 23, and 26 h of propagation time for *Flavobacterium IIb* (C_1), *A. anitratus* (C_2), and *R. nigricans* (H_1), respectively (**Figure 2**). The enzymatic activity at these propagation times showed maximum viscosity reduction in the CMC solution of 77.27, 88.64, and 93.64% for raw extract cellulase enzyme (ϵ) from *Flavobacterium IIb* (C_1), *A. anitratus* (C_2), and *R. nigricans* (H_1), respectively.

The application of the logistic model to the data allows the fit of μ, which in turn is used to describe α and β via least squares and Levenberg−Marquardt methods. Model analysis of enzyme concentration dependence on growth rate during batch fermentations showed that the parameter β was near zero for *Flavobacterium IIb* (C_1) and *R. nigricans* (H_1), indicating that enzyme production for these microorganisms was associated with biomass growth. The parameter α was near zero for *A. anitratus* (C_2); thus, enzyme production by this microorganism was not associated with biomass growth (**Table 1** and **Figure 3**).

Symbiotic or Antagonistic Effects. Mixed Culture Designs. In the previous analysis, experiments were carried out using raw enzyme extract (ϵ) from single cultures. To explore the existence of symbiotic or antagonistic relationships among the different microorganisms in regard to their enzyme production,

Table 1. Characteristic Parameters of the Leudeking–Piret Equation Fitted from Experimental Data

	microorganisms		
	C_1	C_2	H_1
N_0 (g/L)	0.178	0.170	0.110
N_m (g/L)	0.82	0.80	2.50
μ (h^{-1})	0.257	0.287	0.157
(R^2)	0.99	0.99	0.99
α	0.801	0.0	0.594
β	0.036	0.998	0.993
(R^2)	0.99	0.99	0.99

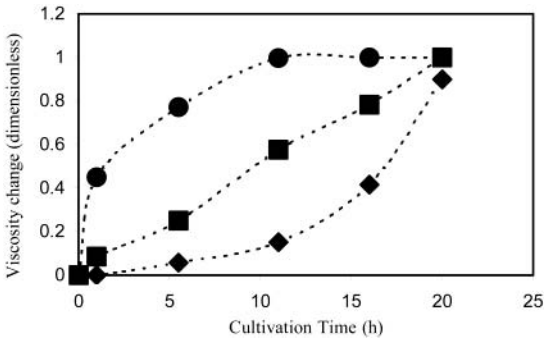

Figure 3. Viscosity change as a function of cultivation time. Nonlinear regression was used for each set of data to determine whether metabolite production is associated [($\beta \rightarrow 0$) for *Flavobacterium IIb* (♦) and *R. nigricans* (●)] or not [($\alpha \rightarrow 0$) for *A. anitratus* (■)] with growth.

Table 2. Experimental Design for Mixed Cultures with the Microorganism with Larger Cellulase Activity

runs/mixture	ϵ_{C1}	ϵ_{C2}	ϵ_{H1}	viscosity (cp)
1	1.0	0	0	340
2	0	1.0	0	190
3	0	0	1.0	10
4	0.5	0.5	0	490
5	0.5	0	0.5	40
6	0	0.5	0.5	50
7	0.333	0.333	0.333	75

we devised a *centroid simplex* design using the raw enzyme extract (ϵ) of the three microorganisms [*Flavobacterium IIb* (C_1), *A. anitratus* (C_2), and *R. nigricans* (H_1)] that exhibited the greatest enzymatic activity. Once again, we evaluated the activity in each experiment as the viscosity change of a CMC solution. **Table 2** presents the final viscosity observed for each experiment within the design.

The analysis of data suggests a special cubic model (the fit was achieved using least squares):

$$[\eta] = 340\epsilon_{C_1} + 190\epsilon_{C_2} + 10\epsilon_{H_1} + 900\epsilon_{C_1C_2} - 540\epsilon_{C_2H_1} - 3315\epsilon_{C_1C_2H_1}$$

In this model, the coefficient for the raw extract of H_1 (ϵ_{H_1}) is significantly different than for the other terms, indicating that the enzyme extract from this microorganism was more effective in achieving cellulose degradation. The coefficient of the nonlinear term involving C_1C_2 is positive; thus, this mixed extract exhibited a synergistic effect. However, the results presented in **Table 2** and **Figure 4** indicate that a mixture of raw extracts from C_1 and C_2 did not reduce viscosity as much as the raw extracts from each microorganism alone. The remaining nonlinear coefficients are all negative, indicating

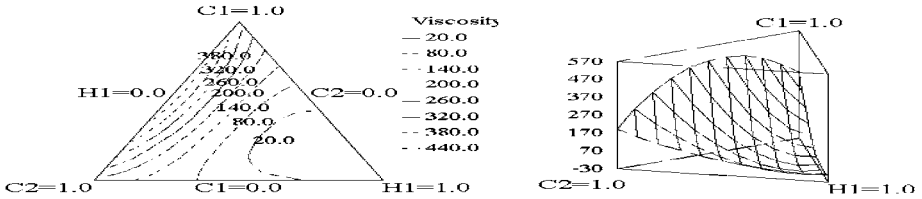

Figure 4. Contours of estimated response surface from the special cubic order model. The lower viscosity is achieved when the mixed extract is composed by $C_1 = 0.19$; $C_2 = 0.13$, and $H_1 = 0.67$.

antagonistic interactions. From the results presented in **Table 2** and **Figure 4**, one can also conclude that the raw extract from H_1 resulted in the largest reduction in viscosity of the CMC solution.

The fitted model is graphically represented in **Figure 4**. The location of the optimum can be accurately computed via standard constrained optimization techniques based on the Newton−Raphson method (*27*). The constraints are as follows:

$$0 \le \epsilon_{C_1} \le 1.0$$

$$0 \le \epsilon_{C_2} \le 1.0$$

$$0 \le \epsilon_{H_1} \le 1.0$$

$$\epsilon_{C_1} + \epsilon_{C_2} + \epsilon_{H_1} = 1.0$$

The solution of the system gives the optimum raw extract composition: $\epsilon_{C_1} = 0.19$, $\epsilon_{C_2} = 0.13$, and $\epsilon_{H_1} = 0.68$. An extract with this composition will maximize cellulase activity, measured via the viscosity reduction of a CMC reference solution.

Ensilage Runs: Screening Mixture Design. The previous results appear to indicate that only three of the originally isolated microorganisms have significant cellulase activity. Ultimately, however, it is total xanthophyll recovery that is important. Thus, we performed an ensilage screening study using all seven microorganisms in a mixture design (**Table 3**). This study attempted to validate the enzymatic activity studies, using total xanthophyll extraction (X_t) from ensilage products as the response variable.

Once again, the data analysis suggests that a compact description of the results can be obtained via fitting a first-order model. However, the analysis of variance of the model

$$[X_t] = 19.86C_1 + 17.1277C_2 + 14.2044C_3 + 16.0312C_4 + 14.9451C_5 + 17.1917H_1 + 12.2842H_2$$

indicates (**Table 4**) that, for a probability distribution value (PV) of 0.1, the model is not a good representation of the data. Even though the model is not a good representation of the data, it can be used to explore the contributions of the different microorganisms in the extraction yield. Note that the coefficients of the model have the following magnitude order: C_1, C_2, H_1, C_4, C_5, C_3, and H_2. Thus, the model suggests that bacteria C_1, C_2, and C_4, as well as the fungus H_1, are the main contributors in the xanthophyll yield extraction. It is important to note that the maximum values of the yield reported (**Table 3**) exhibit a significant increase when compared with the control run. The noted relative importance of C_1, C_2, C_4, and H_1 is confirmed by looking at the individual response of the experiments: runs 8 (C_2, C_4, C_5, H_1; 25% of each one), 16 (C_1, C_2, C_3, H_1; 25% of each one), 17 (C_2, C_3 and C_4; 33% of each one), 20 (C_1, C_4 and C_5; 33% of each one), and 21 (C_1, C_2; 50% of each one), which contain large proportions of the four microorganisms, exhibit the largest total xanthophyll extraction values.

Table 3. Screening Mixture Design for All Microorganisms of Interest for Xanthophylls Extraction

run/ mixture	C_1	C_2	C_3	C_4	C_5	H_1	H_2	$[Xt]_F$[a]
control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	12.97
1	1.0							19.13
2		1.0						14.29
3			1.0					13.03
4				1.0				15.31
5					1.0			15.69
6						1.0		14.93
7							1.0	13.67
8	0	0.25	0	0.25	0.25	0.25	0	21.33
9	0	0	0.25	0	0.25	0.25	0.25	15.43
10	0.25	0	0	0.25	0	0.25	0.25	15.06
11	0	0.334	0	0	0.333	0	0.333	13.03
12	0	0	0.5	0	0	0.5	0	17.46
13	0	0	0	0.5	0	0.5	0	16.50
14	0.5	0	0	0	0.5	0	0	15.91
15	0.333	0.333	0	0	0	0.334	0	14.39
16	0.25	0.25	0.25	0	0	0.25	0	23.35
17	0	0.333	0.333	0.334	0	0	0	22.75
18	0.25	0	0.25	0.25	0.25	0	0	16.69
19	0.14	0.14	0.15	0.14	0.14	0.14	0.15	14.09
20	0.333	0	0	0.334	0.333	0	0	21.47
21	0.5	0.5	0	0	0	0	0	23.24
22	0.166	0.166	0.167	0.166	0.166	0	0.166	13.26
23	0.166	0.166	0.166	0.166	0.167	0.166	0	17.90
24	0.333	0.333	0.334	0	0	0	0	15.75

[a] $[Xt]_F$ = concentration of total xanthophyll in the flour (g of total xanthophylls/kg of flour).

Table 4. Analysis of Variance for the Screening Design[a]

source variation	SS	DF	SM	FR	PV
lineal model	53.76	6	8.96	1.04	0.43
error	146.47	17	8.59		

[a] SS = squares sum, DF = degrees of freedom, SM = squares of the means, FR = Fisher relationship, PV = probability distribution value.

These preliminary observations give the basis to select a reduced set of microorganisms C_1: *Flavobacterium IIb*, C_2: *A. anitratus*, C_4: *E. aerogenes*, and H_1: *R. nigricans* as the most significant in the ensilage process of marigold flower.

Mixed Cultures Optimization in Marigold Ensilage. We performed a second mixture design for the four microorganisms seeking to establish the composition of the culture to be used in the ensilage process. We used a *centroid simplex* design involving pure cultures, as well as all possible combinations of the four microorganisms. Once again, the response variable is total xanthophyll concentration in the product. **Table 5** presents the design and the achieved xanthophyll concentration for each experiment. To facilitate the optimum location, the data are fit

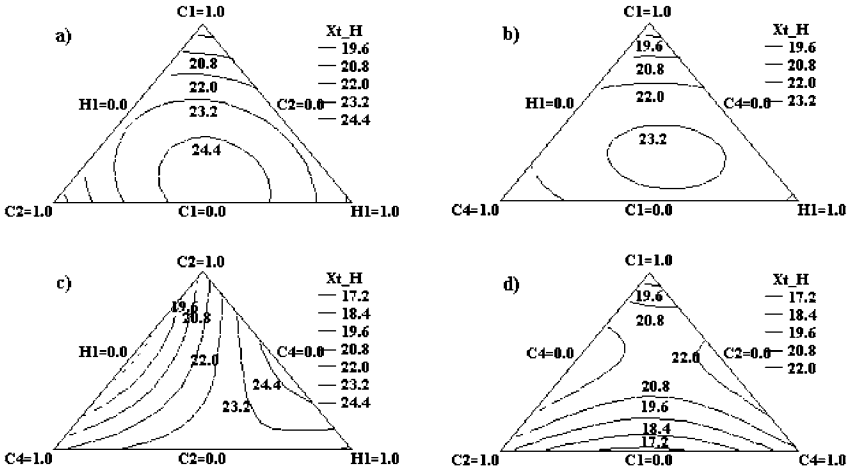

Figure 5. Contours of estimated response surface from the second-order model. Panels c and d indicate an antagonistic effect between C_2: *A. anitratus* and C_4: *E. aerogenes*. The maximum xanthophylls extraction yield is achieved when the concentration of the bacteria C_4 tends to zero and the estimated optimum inoculum starter is composed by $C_1 = 0.12$; $C_2 = 0.40$; $C_4 = 0.0$; and $H_1 = 0.48$.

Table 5. Mixture Design for the *Centroid Simplex* Design[a]

run/mixture	C_1	C_2	C_4	H_1	$[Xt]_F$
control	0.0	0.0	0.0	0.0	12.95
1	1.0	0	0	0	18.88
2	0	1.0	0	0	20.12
3	0	0	1.0	0	20.59
4	0	0	0	1.0	21.62
5	0.5	0.5	0	0	21.86
6	0.5	0	0.5	0	21.93
7	0.5	0	0	0.5	22.67
8	0	0.5	0.5	0	16.66
9	0	0.5	0	0.5	25.17
10	0	0	0.5	0.5	23.78
11	0.333	0.334	0.333	0	22.61
12	0.333	0.333	0	0.334	24.94
13	0.334	0	0.333	0.333	22.60
14	0	0.333	0.334	0.333	20.30
15	0.25	0.25	0.25	0.25	23.42

[a] $[Xt]_F$ = concentration of total xanthophyll in the flour (g of total xanthophyll/kg flower flour).

Table 6. Analysis of Variance for the *Centroid Simplex* Design[a]

source variation	SS	DF	SM	FR	PV
quadratic model	63.39	9	7.04	4.45	0.05
total error	7.92	5			

[a] SS = squares sum, DF = degrees of freedom, SM = squares of the means, FR = Fisher relationship, PV = probability distribution value.

to a second-order polynomial model:

$$[X_t] = 18.693\,C_1 + 20.005\,C_2 + 20.612\,C_4 + 21.769\,H_1 + 14.074\,C_1C_2 + 10.999\,C_1C_4 + 9.542\,C_1H_1 - 13.886\,C_2C_4 + 15.741\,C_2H_1 + 6.796\,C_4H_1$$

The analysis of variance (**Table 6**) shows that this model is a good representation of the data for a probability distribution value (PV) smaller than 0.05. In this model, we observe that the coefficients of the linear terms are very similar, indicating that all four microorganisms by themselves will achieve similar yields. On the other hand, since all nonlinear terms are positive, except the C_2C_4 coefficient, the mixed cultures will exhibit favorable synergic effects. The C_2C_4 interaction can be characterized as antagonistic.

The contours of the fitted model are represented graphically in **Figure 5a−d**. This representation makes the existence of an antagonistic effect more evident between the bacteria C_2: *A. anitratus* and C_4: *E. aerogenes*. In addition, the maximum yield of xanthophyll is achieved when the concentration of the bacteria C_4: *E. aerogenes* approaches zero (**Figure 5c,d**). This result, along with the enzymatic activity study presented previously, leads to the exclusion of the C_4 bacteria from the optimum mixed culture for xanthophyll extraction. The optimum mixed culture should, then, contain C_1: *Flavobacterium IIb*, C_2: *A. anitratus*, and H_1: *R. nigricans* (**Figure 5a**).

Once again, the optimum is estimated via standard constrained optimization techniques, with the following constraints:

$$0 \le C_1 \le 1.0$$

$$0 \le C_2 \le 1.0$$

$$0 \le C_4 \le 1.0$$

$$0 \le H_1 \le 1.0$$

$$C_1 + C_2 + C_4 + H_1 = 1.0$$

The solution of this system gives an estimated optimum inoculum concentration of (C_1: *Flavobacterium IIb* = 0.098; C_2: *A. anitratus* = 0.41; C_4: *E. aerogenes* = 0.0; and H_1: *R. nigricans* = 0.492) and a maximum yield for the xanthophyll extraction of approximately 24.94 g/kg of dry weight (90% more than the control run). This result is similar to that obtained for the cellulase activity studies. In addition, these observations are consistent with the results reported previously (*10*), which demonstrated that the extraction yield was improved when dehydrated marigold flower was treated with a cellulase preparation.

CONCLUSIONS

In this contribution, we examined the efficiency of the ensilage process for xanthophylls extraction from marigold flower. We isolated several microorganisms from natural flora present in the traditional ensilage process. We studied their enzymatic activity and in particular the production of cellulase. From these studies, we conclude that the bacteria *Flavobacterium IIb* and *A. anitratus* and the fungus *R. nigricans* exhibit

significant synthesis and excretion of this enzyme as measured via the reduction in viscosity of a CMC solution. Parallel kinetic studies allowed us to detect the optimum propagation times for each microorganism, which were 16, 23, and 26 h, respectively. These times were selected as the inoculum age to be used in the ensilage studies. The analysis of the enzymatic activity experiments suggest a mixed culture composition given by the following values: C_1: *Flavobacterium IIb* = 15%; C_2: *A. anitratus* = 12%; and H_1: *R. nigricans* = 73% as the starter inoculum.

The ensilage studies, based on mixture experimental designs and response surface methodology, revealed that the inoculum consisting of C_1: *Flavobacterium IIb* = 9.8%; C_2: *A. anitratus* = 41%; and H_2: *R. nigricans* = 49.2% leads to a significant increase in the yield of the total xanthophylls extracted (90% more than the control).

The results of both studies, enzymatic activity and ensilage, suggest that the level of cellulase is the main factor responsible for structural polymer degradation of the cellular walls in the marigold flower petals, facilitating mass transfer and increasing the total xanthophyll extraction.

The differences between the optimum starter inoculum values obtained via the enzymatic activity and ensilage experiments cannot be fully explained by our experimental methodology. These differences may be the result of diffusional phenomena or also could be due to differences in the chemical nature of marigold cell walls (mix of cellulosic and hemicellulosic polymers) as compared to the CMC model. Perhaps other enzymatic activity from the starter cultures contributes to xanthophylls yield in concert with cellulase action.

Our work indicates that the statistical experimental mixture design and the response surface methodology are useful tools for the evaluation of the enzymatic performance of microbial communities.

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