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Microbial interactions for enhancement of α-amylase production by *Bacillus amyloliquefaciens* 04BBA15 and *Lactobacillus fermentum* 04BBA19

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ABSTRACT

Interactions occurring between *Saccharomyces cerevisiae* and two thermostable α-amylase producing strains (*Bacillus amyloliquefaciens* 04BBA15 and *Lactobacillus fermentum* 04BBA19) were analyzed by comparing their growth patterns obtained in isolation with those obtained in mixture. The difference between the patterns was assessed using analysis of variance (ANOVA) in order to measure how much the growth of an organism was affected by other. The results showed two types of interactions in mixed culture; commensalism between *S. cerevisiae* and *B. amyloliquefaciens* 04BBA15 and mutualism between *S. cerevisiae* and *L. fermentum* 04BBA19. In mixed culture, the α-amylase production increased significantly compared to that observed in monoculture ($P < 0.05$). Response surface optimization of fermentation parameters in mixed cultures (initial yeast to bacteria ratio 1.125, temperature 33.5 °C, pH 5.5) resulted in about 1.8 fold higher enzyme production than that observed in the unoptimized fermentation.

1. Introduction

Microorganisms are rarely found in nature as pure cultures and most natural environments are characterized by a great diversity of microbial species interacting in complex ways [14,25]. The growth of a microorganism as a pure culture can be substantially different from its growth in a mixed culture, due to microbial interactions [17]. Such interactions may be synergistic or antagonistic in nature, resulting in enhanced or inhibited proliferation. The antagonistic interactions (antimicrobial interactions) are of particular interest in food microbiology, since they can be used to control the level of pathogenic microorganisms in food products. Synergistic interactions are of great interest for metabolites or enzyme production. In this regard, Leroi and Courcoux [11] demonstrated the stimulation of lactic acid production in *Lactobacillus hilgardii* when this strain was allowed to grow together with *Saccharomyces cerevisiae*. Tremonte et al. [24] showed that synergistic interaction occurring between *L. sakei* and two coagulase negative cocci (CNC) (*Staphylococcus xylosus* and *Kocuria varians*) increased proteolytic activity in these CNC strains.

The interactions between bacterial populations within a community depend on the environmental conditions of the habitat, and under different environmental conditions the same population can exhibit different inter population relationships. The positive interactions between biological populations enhance the ability of the interacting populations to survive within the community in a particular habitat, sometimes enabling whole populations to co-exist in a habitat where individually they cannot exist alone. Since a positive interaction results in an increase in the growth rate of an organism or group of organisms in a particular habitat, such relationship could be used to enhance the production of growth- associated enzymes.

The growth associated-enzymes are the enzymes whose production is primarily linked to the growth of the microorganisms

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producing them. Some starch degrading enzymes such as α-amylases are produced according to this mechanism [2,19,20,22,23].

In this regard, amylases (especially the thermostable ones) constitute a class of enzymes which are of great interest and high demand because of the number of advantages they offer in biotechnology. Amylases have a diverse range of applications that are significant in many fields, such as clinical, medical, and analytical chemistry as well as in the textile, food, fermentation, paper, distillery, and brewing industries [7,8]. The advantages of using thermostable amylases in industrial processes include the decreased risk of contamination, cost of external cooling and increased diffusion rate [19].

The optimal production of a microbial enzyme depends on the nature of the strain involved as well as on the various environmental parameters such as temperature, pH, substrate, and nutrients. Thus, the enhancement of the microbial production of enzymes in general involves optimization of these environmental factors [26]. The improvement of microbial strains by genetic manipulation is another means by which we can also raise the yield of production, especially when this is at industrial scale [15,26]. However, most methods to optimize enzyme production neglect biotic factors such as microbial interactions. Very few studies to date show the impact of biotic factors on the production of enzymes or even metabolites. No previous work has been performed on the co-culture of the above organisms although mixed culture for amylase production has been reported with other strains [1]. Microbial interactions occur only when microbial strains live in community and interact with each other; this justifies the use of mixed cultures to understand the different interactions and their impact on enzyme production, which in our case is a thermostable α-amylase.

The objectives of the present research work were to examine the influence of microbial interactions on the growth and α-amylase production in two amylolytic bacterial strains; and then optimize the production using response surface methodology.

2. Materials and methods

2.1. Microbial strains

Thermostable α-amylase producing bacteria *B. amyloliquefaciens* 04BBA15 and *L. fermentum* 04BBA19 previously isolated from flour waste of a soil sample from Bafoussam, Western region of Cameroon, were used for α-amylase production [21]. The yeast strain *Saccharomyces cerevisiae* from Lesaffre (59703 Marq-France) was used for microbial interaction assessment.

Table 1
Experimental range and levels of the independent variables.

Variables	Symbol coded	Range and levels		
		−1	0	1
Initial yeast to bacteria ratio (R_0)	X_1	0.25	1.125	2
Temperature	X_2	25	33.5	42
pH	X_3	3	5	7

2.2. Determination of yeasts–bacteria interaction

To assess interaction, microbial growth was studied in isolation and in mixture. The generated microbial growth curves were fitted to the model of [3]. The main growth parameter estimated at each combination of pH and temperature were the maximum specific growth rate (μ) and lag phase (λ).

When microorganisms grow together in a mixture, the specific growth rate of the i-th sub-population at time t is:

$$\mu_i(t) = \frac{\frac{d}{dt}xi(t)}{xi(t)} \quad (1)$$

Where $x_i(t)$ is the respective bacterial concentration. The overall concentration is denoted by $x(t) = x_1(t) + x_2(t) \ldots$ (2)

The instantaneous specific growth rate of the whole population, at time t is:

$$\mu(t) = \mu_1(t)\frac{x_1(t)}{x(t)} + \mu_2(t)\frac{x_2(t)}{x(t)} + \ldots \quad (3)$$

Assuming that the fastest growing sub-population does not have a longer lag and smaller starting number than the others, the dominance in rate means numerical dominance in a short time and the specific rate of the whole population becomes practically indistinguishable from the fastest specific growth rate. This justifies the use of the model of [3], to fit growth curves of mixed cultures; the model is based on the assumption that the specific growth rate is practically constant for a phase [17].The difference between the growth rates in isolation and in mixed culture were studied by comparing their models.

Table 2
Box-Behnken design for optimizing α-amylase production in mixed culture.

Run no.	R_0	Temperature	pH	Observed (U mL^{-1})		Predicted (U mL^{-1})	
				I	II	I	II
1	2	33.5	3	96	75.5	98.4	142.35
2	1.125	25	7	216	197	215.45	219.21
3	2	25	5	184	123	183.05	142.35
4	1.125	33.5	5	360	302	357.6	325.69
5	0.25	25	5	184	114	186.95	129.47
6	2	33.5	7	141.6	121	281.5	182.13
7	0.25	33.5	3	168	134	248.5	142.03
8	1.125	33.5	5	350.4	299	357.6	249.61
9	1.125	25	3	256	221	254.55	218.48
10	1.125	42	7	272	245	273.45	212.9
11	0.25	42	5	168	112	168.95	117
12	0.25	33.5	7	117.6	85	115.2	167.2
13	1.125	33.5	5	360	300	357.6	131.95
14	1.125	42	3	184	134	184.55	325.69
15	1.125	33.5	5	357.6	302	357.6	290.41
16	1.125	33.5	5	360	300	357.6	286.25
17	2	42	5	192	121	189.05	193.97

I mixed culture I, II mixed culture II.

Table 3
Growth parameters in pure and mixed culture of *B. amyloliquefaciens* 04BBA15 and *S. cerevisiae* at 30, 35, and 40 °C in the presence of starch broth.

		Pure culture		Mixed culture	
Temperature (°C)	Strain	μ_{max} (h^{-1})	lag (h)	μ_{max} (h^{-1})	lag (*h*)
30	*B. amyloliquefaciens*	0.142±0.003[a]	3.302±0.005[a]	0.142±0.003[a]	3.304±0.005[a]
	S. cerevisiae	0.105±0.002[a]	6.602±0.003[a]	0.188±0.001[b]	0.802±0.007[b]
35	*B. amyloliquefaciens*	0.214±0.003[a]	2.234±0.002[a]	0.214±0.002[a]	2.234±0.005[a]
	S. cerevisiae	0.180±0.001[a]	4.058±0.004[a]	0.450±0.004[b]	1.532±0.004[b]
40	*B. amyloliquefaciens*	0.350±0.004[a]	1.327±0.005[a]	0.350±0.003[a]	1.331±0.001[a]
	S. cerevisiae	0.230±0.001[a]	3.101±0.001[a]	0.345±0.00[b]	2.054±0.003[b]

μ_{max} is the maximum growth rate in h^{-1} and lag is the duration of lag phase in hours (h).
[a] For each microbial strain the average with the different superscriptson the same row for pure and mixed culture are significantly different ($P < 0.05$).
[b] For each microbial strain the average with the different superscriptson the same row for pure and mixed culture are significantly different ($P < 0.05$).

2.3. Monoculture fermentation

The microbial strains (*B. amyloliquefaciens* 04BBA15, *L. fermentum* 04BBA19, *S cerevisiae*) were respectively purified by subculture on Nutrient, de Man Rogosa and Sharpe (MRS) and Sabouraud agar. A 24 h old colony of each strain was inoculated in 100 mL Erlenmeyer flask containing 50 mL of Nutrient broth (Liofilchem s.r.l. Bacteriology products) and incubated at 30 °C for 24 h in a rotary shaker (Kotterman, Germany) with a speed of 150 rpm.

Spectrometry followed by the plate counting method was used to determine microbial concentration of the inoculum in CFU mL^{-1}. Different dilutions of the inoculum were prepared aseptically and their optical densities were measured at 600 nm; 0.1 mL of the various dilutions of the inoculum were then spread on the surface of the plate counting agar (PCA) (Liofilchem s.r.l. Bacteriology products) and incubated for 24 h at 30 °C to determine the microbial concentration of the inoculum in CFU mL^{-1}. A standard curve of optical density as a function of microbial count was also used to calculate the inoculum concentration in CFU mL^{-1}.

To run the fermentation, 1 mL of each inoculum containing 10^6 CFU mL^{-1} after keeping for 24 h was introduced aseptically into 500 mL Erlenmeyer flask containing 250 mL of a broth composed of 1% (w/v) of soluble starch (which plays the role of amylase inducer) supplemented with 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.05% (w/v) magnesium sulphate heptahydrate. The Erlenmeyer flasks were incubated in a rotary shaker (Kotterman) at 30 °C, 150 rpm for 3 days. The kinetic of growth was studied by measurement of microbial load in each fermenting broth at a regular time interval (10 h) for a total incubation time of 70 h. Every 10 h, an aliquot of 0.5 mL of fermenting broth was picked aseptically for microbial enumeration. The 10-fold serial dilution and pour plate method on Sabouraud's agar supplemented with 0.1 mg L^{-1} chloramphenicol was used for enumeration of *S. cerevisiae* whereas *B. amyloliquefaciens* 04BBA15and *L. fermentum* 04BBA19 were enumerated respectively on nutrient and de Man Rogosa and Sharpe (MRS) (Liofilchem s.r.l. Bacteriology products) agars using the same method. Each experiment was carried out in triplicate.

2.4. Mixed culture fermentation

Two different mixed cultures were carried out for the assessment of microbial interaction. The first mixed culture (mixed culture I) involved the simultaneous culture of *S. cerevisiae* and *B. amyloliquefaciens* 04BBA15, while the second (mixed culture II) involved a simultaneous culture of *S. cerevisiae* and *L. fermentum* 04BBA19. To run the fermentation, 0.5 mL of 24 h old yeast inoculum and 0.5 mL of 24 h old bacteria inoculum containing 1.0×10^6 CFU mL^{-1} were aseptically mixed in 250 mL of culture broth (with the same composition as above for the monoculture fermentation) in 500 mL Erlenmeyer flasks. The whole was incubated at 30 °C, 150 rpm.

For microbial enumeration in mixed culture I, the total microbial load and the yeast load (*S. cerevisiae*) were respectively determined using the pour plate method on plate counting agar (PCA) and Sabouraud's agar supplemented with 0.1 mg L^{-1} of chloramphenicol, then *B. amyloliquefaciens* 04BBA15 load was deduced by subtraction of *S. cerevisiae* load from the total microbial load.

Regarding the mixed culture II, a differential medium (MRS-Starch-Bromocresol-purple agar) was developed for enumeration of *L. fermentum* 04BBA19. This medium allowed differentiation of *S. cervevisiae* and *L. fermentum* 04BBA19. After 24 h of culture at 30 °C, the colonies of *L. fermentum* 04BBA19 were differentiated from the colonies of *S. cerevisiae*, by the fact that they were able to produce lactic acid from starch during incubation, and this acidification was materialized by a yellow halo around the colonies. However *S. cerevisiae* colonies could not display yellow halos on this medium. The composition of MRS-starch-bromocresol purple was: 1% (w/v) soluble starch, 1% (w/v) peptone, 0.5% (w/v) yeast extract, 1% (w/v) beef extract, 0.02%
(w/v) magnesium sulphate heptahydrate, 0.005% (w/v) manganese sulphate tetrahydrate, 1% (w/v) Tween 80, 0.5% sodium acetate trihydrate, 0.2% (w/v) triammonium citrate, 0.2% (w/v)

Table 4
Growth parameters in pure and mixed culture of *L. fermentum* 04BBA19 and *S. cerevisiae* at 30, 35, and 40 °C in the presence of starch broth.

		Pure culture		Mixed culture	
Temperature (°C)	Strain	μ_{max} (h^{-1})	lag (h)	μ_{max} (h^{-1})	lag (h)
30	*L. fermentum*	0.163±0.001[a]	5.574±0.005[a]	0.209±0.001[b]	3.203±0.003[b]
	S. cerevisiae	0.105±0.001[a]	6.645±0.005[a]	0.140±0.001[b]	1.453±0.001[b]
35	*L. fermentum*	0.257±0.001[a]	4.742±0.005[a]	0.407±0.001[b]	0.132±0.001[b]
	S. cerevisiae	0.205±0.001[a]	4.752±0.005[a]	0.781±0.001[b]	0.102±0.002[b]
40	*L. fermentum*	0.450±0.001[a]	3.722±0.005[a]	0.704±0.001[b]	0.099±0.001[b]
	S. cerevisiae	0.101±0.001[a]	4.051±0.005[a]	0.185±0.001[b]	3.673±0.005[b]

μ_{max} is the maximum growth rate in h^{-1} and *lag* is the duration of lag phase in hours (h).
[a] For each microbial strain the average with the different superscripts on the same row for pure and mixed culture are significantly different ($P < 0.05$).
[b] For each microbial strain the average with the different superscripts on the same row for pure and mixed culture are significantly different ($P < 0.05$).

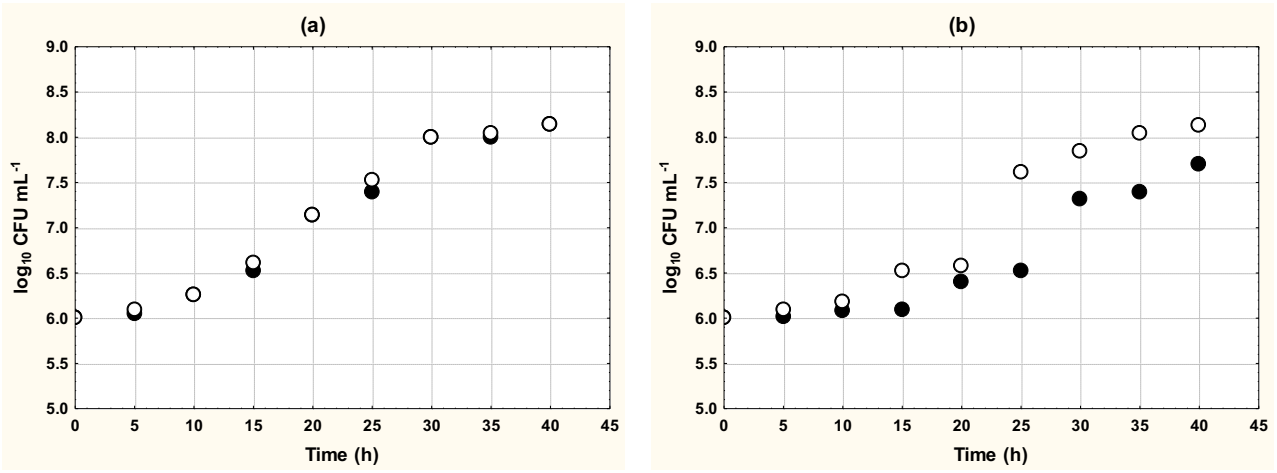

Fig. 1. Profile of growth in pure (●) and mixed culture (○) for *B. amyloliquefaciens* 04BBA15 (a), and *S. cerevisiae* (b) in starch broth at 30 °C.

dipotassium hydrogen phosphate 0.1% (w/v) bromocresol purple; 1% (w/v) agar.

2.5. α-Amylase assay

For the determination of α-amylase production during pure and mixed cultures, the fermented broth was centrifuged at 8000 g, 4 °C for 30 min. The cell free supernatant was recovered as a crude enzyme extract. The activity of α-amylase was assayed using a modified method of [9]. In a typical run, 5 mL of 1% (w/v) soluble starch solution and 2 mL of 0.1 mol L^{-1} phosphate buffer (pH 6.0) were mixed and maintained at 60 °C for 10 min, then 0.5 mL of appropriately diluted enzyme solution was added. After 30 min the enzyme reaction was stopped by rapidly adding 1 mL of 1 mol L^{-1} HCl into the reaction mixture. For the determination of residual starch, 1 mL of the reaction mixture was added to 2.4 mL of an iodine solution containing 3% (w/v) KI, 0.3% (w/v) I_2 diluted to 4% (v/v) and its optical density was read at 620 nm using a spectrophotometer (Secoman). A standard curve for optical density as a function of starch concentration was used to determine starch concentration. One unit of α-amylase activity (U) was defined as the amount of enzyme able to hydrolyze 1 g of soluble starch in 60 min under the experimental conditions. All the values presented are means of three replicates.

2.6. Optimization of α-amylase production in mixed culture

The optimization of α-amylase in mixed culture was focused on three important independent variables, the initial yeast to bacteria ratio (R_0), the temperature (T) and the pH. A Box–Behnken design with five replicates at the central point resulting in 17 experiments generated by Design Expert 8.0 software was used [5]. Each independent variable was studied at three different levels (low, medium, and high, coded as −1, 0, and +1, respectively). The coded variables are shown in Table 1 and the experimental design is shown in Table 2. All the experiments were done in triplicate and the average of α-amylase production obtained was taken as the dependent variable or response (Y_i).

The second order polynomial coefficients were calculated and analyzed using the Design Expert 8.0 software. The general form of the second order polynomial equation is:

$$Y_i = \alpha_0 + \sum \alpha_i X_i + \sum \alpha_{ii} X_i^2 + \sum \alpha_{ii} X_i X_j \quad (4)$$

Where Y_i is the predicted response, $X_i X_j$ are input variables which influence the response variable Y; α_0 is the offset term; α_i is the ith linear coefficient; α_{ii} the ith quadratic coefficient and α_{ij} is the ijth interaction coefficient.

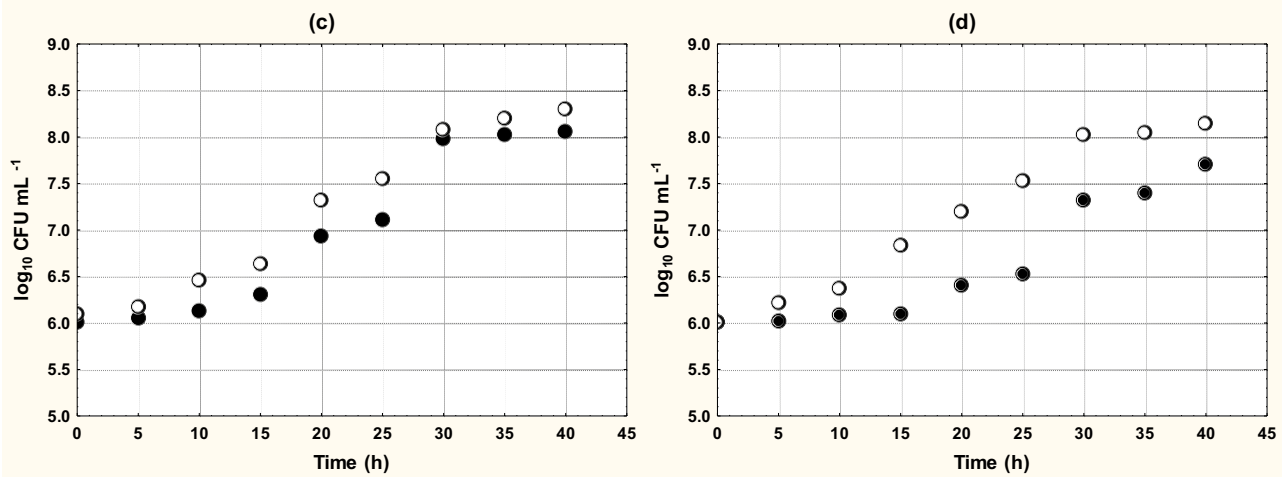

Fig. 2. Profile of growth for pure (●) and mixed culture (○) of *L. fermentum* 04BBA19 (c), and *S. cerevisiae* (d) in the starch broth at 30 °C.

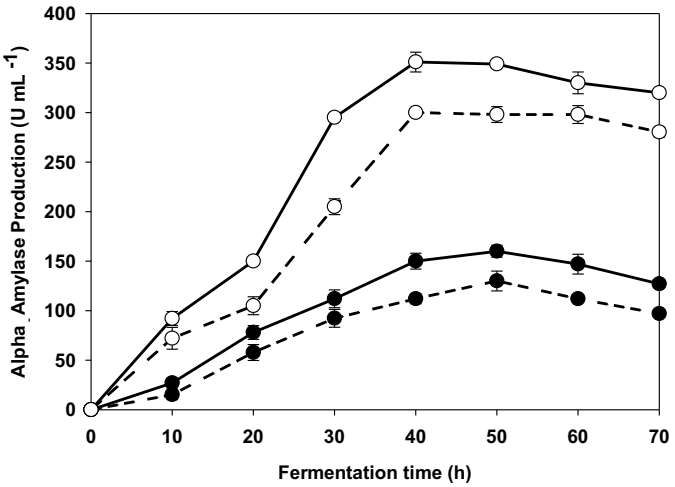

Fig. 3. α-Amylase production for pure (●) and mixed culture (○) at 30 °C in starch broth by *B. amyloliquefaciens* 04BBA15 (▬▬) and *L. fermentum* 04BBA19 (▬ ▬ ▬). The two amylase producing strains were first cultivated individually, and then were respectively mixed with *S. cerevisiae* (which is unable to produce amylase). The α-amylase production was evaluated by measuring α-amylase activity in cell free supernatant obtained from mixed culture fermentation. The initial yeast to bacteria ratio was 1.

2.7. Statistical analyses

In order to confirm effective interaction between studied microbial strains, the ANOVA of growth parameters μ_{max} and N_{max} when passing from pure to mixed culture was performed. This analysis included the Fisher's *F*-test and its associated probability *p* (*F*). All these statistical analyses were carried out using a computer's program Design Expert 8.0.

3. Results and discussion

3.1. Kinetics of fermentation and evidence of microbial interactions

The microbial strains propagated in culture broth according to a usual profile including lag, exponential and stationary phases. The maximum specific growth rate (μ_{max}) and lag time of each strain in starch broth at 30 °C in mono and mixed cultures were derived by the curve fitting procedure of Baranyi and Robert [3]. The values of μ_{max} and lag time were 0.142 h^{-1}; 3.302 h, 0.163 h^{-1}; 5.574 h, 0.105 h^{-1}; 6.445 h (average of three replications) respectively for *B. amyloliquefaciens* 04BBA5, *L. fermentum* 04BBA19 and *S. cerevisiae*. These kinetics parameters, their standard deviations and the ANOVA are summarized in Tables 3 and 4.

The first mixed culture (mixed culture I) involved *B. amyloliquefaciens* 04BBA15 and *S. cerevisiae*. The comparison of the profile of growth for pure and mixed cultures (Fig. 1a and b) showed that when *B. amyloliquefaciens* 04BBA15 was growing together with *S. cerevisiae*, the growth curve of *S. cerevisiae* shows a significant rise while the growth of *B. amyloliquefaciens* 04BBA15 remained unchanged. This observation suggests that there is an interaction between the both microbial populations when they coexist in mixed culture, since the microbial interaction is defined as the effect of one population on the other [6,17]. This interaction was classified as a positive one, especially a commensalism owing to the fact that the presence of *B. amyloliquefaciens* 04BBA15 stimulated the growth of *S. cerevisiae*, while the growth of *S. cerevisiae* did not affect the growth of *B. amyloliquefaciens* 04BBA15. Commensalism is generally defined as a relationship between members of different species living in proximity (the same cultural environment) in which one organism benefits from the association but the other is not affected (Peclczar et al., 1993) [16,18]. The commensalism between *B. amyloliquefaciens* and *S. cerevisiae* can be explained by the fact that *B. amyloliquefaciens* is capable of hydrolyzing starch present in the culture medium. This hydrolysis results in the release into the culture medium of glucose which yeast *S. cerevisiae* needed for effective growth. The study of the growth of *S. cerevisiae* in single culture showed that in the starch broth (medium composed of 1% (w/v) of soluble starch 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.05% (w/v) magnesium sulphate heptahydrate), this strain utilizes only peptone and yeast extract for growth but is unable to utilize the starch, while in mixed culture it benefits of glucose produced as a result of the hydrolysis of starch by the bacterial strain. The growth of *S. cerevisiae* in mixed culture is comparable to its growth in pure culture in the presence of glucose as carbon source.

Leroi and Courcoux [11] found a similar interaction between *S. florentinus* and *Lactobacillus hilgardii*. Benjamas et al. [4] also found the stimulation of growth of *L. kefirafaciens* by *S. cerevisiae*. Pin and Baranyi [17] compared the growth response of some groups of bacteria found on meat as a function of the pH and temperature when grown in isolation and grown together. They used a statistical *F* test to show if the difference in the growth rates

Table 5
Analysis of variance (ANOVA) for the quadratic model for mixed culture I.

Source	Sum of squares	df	Mean square	*F* value	*P*-value prob > *F*	
Model	124597.5	9	13844.16	887.7716	7.37E-10	Significant
X_1	131.22	1	131.22	8.414621	0.02296	
X_1	72	1	72	4.617076	0.068742	
X_1	1240.02	1	1240.02	79.51759	4.53E-05	
X_1 X_2	144	1	144	9.234152	0.01888	
X_1 X_3	25027.24	1	25027.24	1604.898	1.57E-09	
X_2 X_3	4096	1	4096	262.6603	8.29E-07	
X_1^2	51737.78	1	51737.78	3317.74	1.25E-10	
X_2^2	17652.89	1	17652.89	1132.01	5.31E-09	
X_3^2	15590.41	1	15590.41	999.7515	8.18E-09	
Residual	109.16	7	15.59429			
Lack of Fit	40.04	3	13.34667	0.772377	0.566853	Not significant
Pure error	69.12	4	17.28			
Cor total	124706.6	16				

CV = 1.60; R^2 = 0.999.

Table 6
Analysis of variance (ANOVA) for the quadratic model for mixed culture II.

Source	Sum of squares	df	Mean square	*F* value	*P*-value prob > *F*	
Model	78137.03	9	8681.892	5.914249	0.014343	Significant
X_1	996.0869	1	996.0869	0.678551	4.37E-01	
X_2	9902.774	1	9902.774	6.745935	0.035565	
X_3	11152.16	1	11152.16	7.597036	0.028247	
X_1 X_2	6100.168	1	6100.168	4.155536	8.09E-02	
X_1 X_3	3989.69	1	3989.69	2.717843	0.143222	
X_2 X_3	95.0625	1	95.0625	0.064758	8.06E-01	
X_1^2	51830.75	1	51830.75	35.30797	5.75E-04	
X_2^2	4554.695	1	4554.695	3.102734	1.22E-01	
X_3^2	9995.359	1	9995.359	6.809005	3.49E-02	
Residual	10275.73	7	1467.962			
Lack of Fit	7169.012	5	1433.802	0.923033	0.593448	Not significant
Pure error	3106.72	2	1553.36			
Cor total	88412.76	16				

CV = 18.73; R^2 = 0.884.

in mixed cultures was significant. Malakar et al. [12] quantified the interactions between *L. curvatus* and *Enterobacter cloacae* in broth culture using a set of coupled differential equations. Malakar et al. [13] quantified the interactions of *L. curvatus* cells in colonies using a coupled growth and diffusion equation.

Most of the studies focused their attention on the impact of interactions on the growth of different microbial communities but very few dealt with the impact of microbial interactions on enzymes or metabolites production.

In the second mixed culture (mixed culture II) involving *L. fermentum* 04BBA19 and *S cerevisiae*, (Fig. 2c and d), the growth curve of the both microbial strains were different from that obtained in pure culture. A significant increase in logcount ($\log_{10}$CFU mL^{-1}) was observed for each of these two strains when they grew together. This indicates that there is a positive interaction between *L. fermentum* 04BBA19 and *S cerevisiae*. The rise of the both microbial population means that there is mutualism between *L. fermentum* 04BBA19 and *S. cerevisiae.* Mutualism defines the relationship in which some reciprocal benefit accrues to both partners [18]. Mutualism between *S. cerevisiae* and *L. fermentum* 04BBA19 could be explained by the fact that the amylolytic lactic acid bacterium strain *L. fermentum* through its amylase activity releases the glucose necessary for the growth of yeast. The yeast *S. cerevisiae* in turn stimulates amylase activity in *L. fermentum* by consuming a portion of the glucose produced, thus reducing any excess glucose. The excess glucose is generally recognized as a repressor of the enzyme synthesis in many bacteria [10].

The analysis of variance (ANOVA) of growth parameters (μ_{max} and lag) of mixed culture I showed that for *B. amyloliquefaciens* 04BBA15, there was no significant differences ($P < 0.05$) between these parameters in pure and mixed culture (Table 3), while for *S. cerevisiae*, significant changes ($P < 0.05$) in growth parameters were observed when both strains were grown together, thus confirming the occurrence of an interaction between the two microorganisms. On the other hand the kinetic parameters varied with temperature, the maximum of interaction between *S. cereviae* and *B. amyloliquefaciens* 04BBA15 was achieved at 35 °C (Table 3). At this temperature, the specific maximum growth rate of *S cerevisiae* increased considerably and reached 0.450 h^{-1}. An important reduction of lag time was observed and the logcount of *S. cerevisiae* reached maximum value (12.0), indicating that temperature was an important environmental factor affecting the interaction between both microorganisms.

The ANOVA of growth parameters of mixed culture II (Table 4) also showed significant differences ($P < 0.05$) for all the growth parameters when passing from pure to mixed culture. This significant change confirmed the existence of positive interaction between *L. fermentum* 04BBA19 and *S. cerevisiae*.

3.2. Influence of microbial interactions on α-amylase production

In monoculture fermentation (pure culture), the level of amylase production increased and reached maximum value after 40–50 h of incubation at 30 °C, 107.5 ± 0.5 U mL^{-1} and 147.5 ± 0.3 U mL^{-1} respectively for *B. amyloliquefaciens* 04BBA15 and *L. fermentum* 04BBA19. When each of these cultures was associated with *S. cerevisiae*, a significant increase in α-amylase production was observed in the culture medium (Fig. 3). The duration for maximal amylase production (30 h) was less than that observed in pure culture. The levels of amylase production were 300.0 ± 0.3 U mL^{-1} and 351.1 ± 0.4 U mL^{-1} respectively for *B. amyloliquefaciens* 04BBA15and *L. fermentum* 04BBA19 (Fig. 3). The presence in the

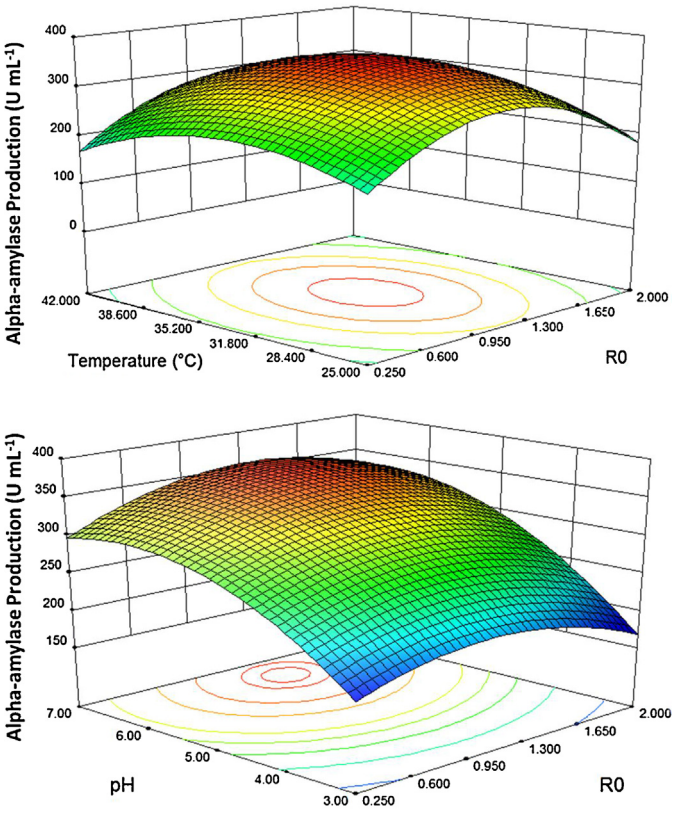

Fig. 4. Response surface and contour plot for α-amylase production as a function of R_0 (initial yeast to bacteria ratio), temperature and pH in mixed culture I (*B. amyloliquefaciens* 04BBA15 and *S. cerevisiae*).

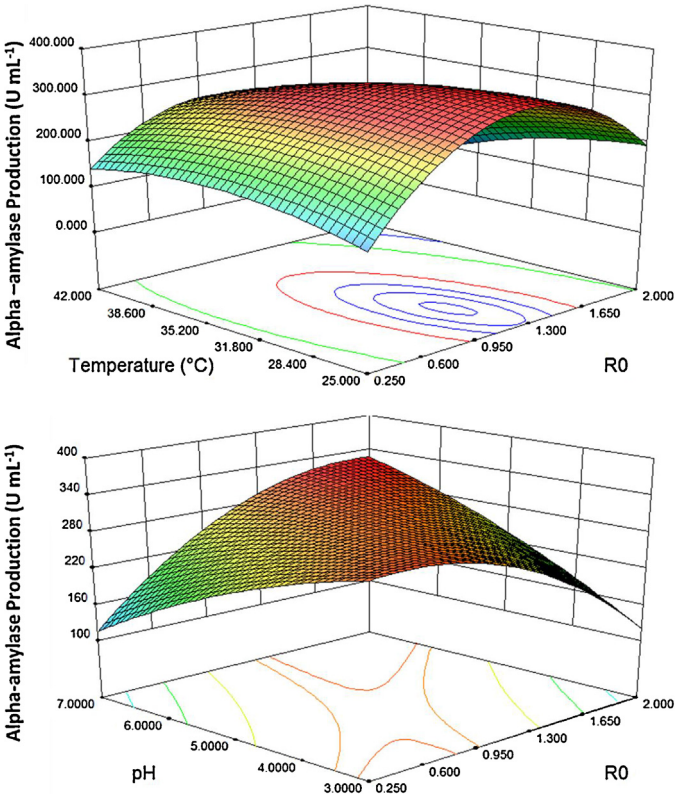

Fig. 5. Response surface and contour plot for α-amylase production as function of R_0 (initial yeast to bacteria ratio), temperature and pH in mixed culture II (*L. fermentum* 04BBA19 and *S. cerevisiae*).

culture medium of *S. cerevisiae* stimulated and enhanced α-amylase synthesis by both amylase producing bacteria. Additionally to the effect of mixed culture on microbial growth (Fig. 2c and d), this observation is proof of the existence not only of a commensalism, but a synergism between *B. amyloliquefaciens* and *S. cerevisiae*. Synergism is regarded as the ability of two or more organisms to bring about changes (usually chemical) that neither can accomplish alone [16]. The same kind of synergism may also exist between *L. fermentum* 04BBA15 and *S. cerevisiae*, since there was a rise of α-amylase production when the two strains were cultivated together. Synergism in both cases could be explained by the fact that in starch broth *B. amyloliquefaciens* 04BBA15 and *L. fermentum* 04BBA19 hydrolyze starch which leads to the increase in glucose or other oligosaccharids that the yeast *S. cerevisiae* needs for a normal growth since it is unable to convert starch into glucose. Part of the glucose release through starch hydrolysis is immediately utilized by *S. cerevisiae.* The increase in α-amylase production could be attributed to the rapid consumption of glucose by both organisms.

3.3. Optimization of α-amylase production in mixed culture

The Box–Behnken design was used to study the interactions among significant factors (initial yeast to bacteria ratio R_0, temperature, pH) and also determine their optimal levels. The symbol coded of the variables, the range and level are presenting in Table 1. The results are represented in Table 2.

Multiple regression analysis was used to analyze the data and a polynomial equation was derived from regression analysis for the mixed culture I and mixed culture II. The final equations in term of coded factors are summarized in the Eqs. (5) and (6) respectively for mixed culture I and II.

$$Y_i = 357.60 + 4.05X_1 - 3.00X_2 + 12.45X_3 + 6.00X_1X_2 + 79.10X_1X_3 + 32.00X_2X_3 - 110.85X_1^2 - 64.75X_2^2 - 60.85X_3^2 \quad (5)$$

$$Y_i = 325.69 - 12.43X_1 - 38.39X_2 + 38.76X_3 - 50.91X_1X_2 + 75.06X_1X_3 + 4.88X_2X_3 - 170.92X_1^2 - 37.69X_2^2 - 74.04X_3^2 \quad (6)$$

The equations in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels of the factors are coded as −1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

The statistical model was checked by *F*-test, and the analysis of variance (ANOVA) for the response surface quadratic model is summarized in Tables 5 and 6. The Model *F*-value of 887.77 and 5.914 imply that the two models used for mixed culture I and mixed culture II are significant. There is only a 0.01% and 1.43% chance that an *F*-value could occur due to noise. Values of "Prob > *F*" less than 0.0500 indicate model terms are significant. For the first model corresponding to mixed culture I, X_1, X_3, X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2, X_3^2 are significant model terms whereas in the case of the second model corresponding to mixed culture II, only X_2, X_3, X_1^2, X_3^2 are significant. Values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit *F*-value" of 0.77 and 0.923 respectively for mixed culture I and II imply the Lack of Fit is not significant relative to the pure error. There is a 56.69% and 59.34 chance respectively for mixed culture I and mixed culture II that a "Lack of Fit *F*-value" could occur due to noise. Non-significant Lack of Fit is good. The R^2 value (multiple correlation coefficient) closer to 1 for both mixed culture indicates better correlation between the observed and predicted values. The coefficient of variation (CV) indicates the degree of precision with which the experiments are compared. The lower reliability of the experiment is usually indicated by a high value of CV. In the present cases a low CV (1.60 and 18.73) denotes that the experiments performed are highly reliable. The *P* value denotes the significance of the coefficients and is also important in understanding the pattern of the mutual interactions between the variables. For mixed culture I the *P* values (Table 5) suggest that among the three variables studied, X_1 (initial yeast to bacteria ratio) and X_3 (pH) showed maximum interaction, while in the mixed culture II the variable, X_1 (initial yeast to bacteria ratio) and X_2 (temperature) showed maximum interaction.

The cumulative effect and optimal levels of the variables were determined by plotting the response surface curves. The response surface curves are represented in Figs. 4 and 5. These figures represent the interactive effect of initial yeast to bacteria ration (R_0), temperature, and pH on α-amylase production.

3.4. Validation of the models

Validation of the models for both mixed cultures was carried out under conditions predicted by the two models. A close correlation was seen between the experimental and predicted values. The optimal levels of the process variables for α-amylase production were initial yeast to bacteria ratio (1.125), temperature (33.5 °C) and pH (5.0).

4. Conclusion

This study demonstrated the stimulation of α-amylase production in two bacteria (*B. amyloliquefaciens* 04BBA15, and *L. fermentum* 04BBA19) by the yeast *S. cerevisiae*. The study highlighted the impact of microbial interactions (symbiosis) on microbial enzyme production, especially the thermostable α-amylase. Significant enhancement of α-amylase production was observed when the enzyme producing strains were cultured together with *S. cerevisiae*. Microbial interactions were important events influencing enzyme synthesis in mixed culture; hence the initial microbial ratio was among the highly significant factors for α-amylase production. Taking into account these biotic factors, the optimization of enzyme production led to a high level of enzyme in mixed culture, thus microbial interactions could be recommended for use in the enhancement of industrial microbial enzyme production.

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