academicJournals

Vol. 15(27), pp. 1429-1441, 6 July, 2016
DOI: 10.5897/AJB2016.15323
Article Number: 3DAD01559419
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Optimization of biomass production of *Acetobacter pasteurianus* KU710511 as a potential starter for fruit vinegar production

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Received 6 March, 2016; Accepted 18 May, 2016

The objective of the present work was first the isolation of novel acetic acid bacteria strains from natural Moroccan habitats, and then, the evaluation of their ability to produce microbial starters for vinegar production on a large scale. The strains were isolated from figs, dates, cactus, and traditional fruit vinegars. Four strains, selected from a total of 63 isolates, were confirmed as belonging to *Acetobacter* species according to biochemical and molecular studies based on 16s rRNA sequence analysis. Acetous fermentation tests, performed on date and apple fermented juices using selected *Acetobacter* strains, showed a high capacity of acidification. The most efficient strain KU710511, isolated from Morrocan cactus (*Opuntia ficus-indica*), was identified as *Acetobacter* strain closely related to *A. pasteurianus* and yielded 42.5 g/L acidity in apple juice. Cell growth optimization was carried out for KU710511 using response surface methodology (RSM). The linear, quadratic, and interaction effects of four factors-ethanol, acetic acid, glucose concentrations and pH-were studied by the application of a central composite design. Thirty experiments were designed to predict the maximum concentration of cell biomass. The optimal calculated values of ethanol, acetic acid, glucose and pH allowing the prediction of the maximum biomass production (2.21 g/L) were 28.18, 10.12, 15.15 and 5.33 g/L, respectively. Subsequently, further batch fermentations were carried out in a 6-L lab-bioreactor at optimal conditions. The results were in line with the predicted values. It can be concluded that the studied strain is well suited to be used as a parental strain to prepare a starter for fruit vinegar production.

Key words: Isolation, vinegar, starter, *Acetobacter*, acetic fermentation, response surface methodology.

INTRODUCTION

Acetic acid bacteria (AAB) is a group of microorganisms known to have unique fermentation ability, so-called "oxidative fermentation," a process where ethanol is converted to acetic acid (Saichana et al., 2014). They are

ubiquitous organisms that are well adapted to sugar and ethanol rich environments (Bartowsky and Henschke, 2008). Vinegar, from the French vin aigre, meaning “sour wine,” can be made from almost any fermentable carbohydrate source, including apples, dates, grapes, pears, coconut, honey, etc. (Johnston and Gaas, 2006).

Morocco is one of the main fruit and vegetable producing countries on the southern shore of the Mediterranean Sea. Thousands of tons are commercialized annually in the national and international market. According to a citrus packing stations association, 30 to 40% of estimated losses of fruits and vegetables are generated annually on the production sites and from the processing plants (L’economiste, 2010). These substandard fruits are, in most cases, improper to be commercialized on the local market. Thus, transforming them via biotechnological processes to obtain exotic products with the local knowledge is, therefore, essential (Benkerroum, 2013; Ndoye et al., 2007a).

Vinegar is a product of high nutritional and cultural value (Johnston and Gaas, 2006). It is obtained from double stage fermentation, alcoholic and acetic, performed, respectively, by yeasts and acetic acid bacteria. The use of traditional processes, where both fermentation steps are performed spontaneously, generates improper vinegar because of the possibility to contain, among other substances, mycotoxins. The presence of these compounds in food is of great concern for human health due to their properties to induce severe toxicity effects at low dose levels (Fernández-Cruz et al., 2010).

In order to produce biological type vinegar that meets the chemical and microbiological standards, it was necessary to select endogenous strains from Moroccan natural habitats, which are able to perform efficient acetic acid fermentation. In fact, until recently, the production of industrial vinegar in Morocco has required industrial imported starter cultures. Obtaining valid starters from local screened bacteria will help to avoid the need to import them. AAB are obligate aerobes and their growth is highly dependent upon the availability of a carbon source, nitrogen source, molecular oxygen, and growth factors (Hidalgo et al., 2010; Liu et al., 2008). Many physicochemical factors are observed as influencing the growth and productivity of AAB. pH of the fermenting must, temperature, and the concentration of ethanol are, beside dissolved oxygen, the main factors that influence the growth of AAB (Drysdale and Fleet, 1988). Furthermore, glucose acts as a principal carbon source for most strains of AAB (Guillamón and Mas, 2009). In addition, it was demonstrated previously that acetic acid would have a stimulatory effect on cell growth of AAB at low concentration (De Ory et al., 2004).

Response surface methodology (RSM) has been extensively used for optimization of medium composition and conditions of fermentation (Cui et al., 2006). It has been reported that this method is more effective compared to conventional techniques, which are extremely laborious and time-consuming. Furthermore, conventional methods do not guarantee the determination of optimal conditions and are unable to detect interactions between two or more factors (Cui et al., 2006; Liu et al., 2008). In this study, RSM was selected in order to search for the crucial influencing growth factors and to exhibit their synergistic interactions for biomass production of selected Acetobacter strains. Thus, the objective of this work was, firstly, the isolation of novel acetic acid bacteria from a variety of Moroccan foods (fruits and vinegars) and the identification of endogenous isolates, which can be used for the production of bacterial starters. Secondly, an examination of their performance and the optimization of cell growth depending on the growth factors were performed using RSM.

MATERIALS AND METHODS

Samples and microorganisms

Samples used for isolation of AAB were recovered from different regions of Morocco and grouped into two groups: (1) Fruits including apple (*Malus domestica;* variety *Golden delicious*, from Midelt), dates (*Phoenix dactylifera*; variety *black Bousthami*, from Zagora), figs (*Ficus carica* L. from Ouarzazate) and cactus (*Opuntia ficus-indica*; *Moussa*, from Sidi Ifni); (2) Vinegars: Namely apple vinegar originating from Midelt and cactus vinegar from Ait Baamrane, both manufactured in a traditional manner. Immediately after collection, the samples were stored at a low temperature (4°C) to protect them from deterioration.

Isolation procedure

Fruit samples were cut into small slices and transferred in a GYEA enrichment medium that consisted of glucose 2% (w/v), yeast extract 1% (w/v), ethanol 2% (v/v) and acetic acid 1% (v/v). Samples were incubated under agitation (120 rpm) at room temperature (25-30°C) for one week. Then, 0.2 ml of liquid samples were diluted and inoculated in modified YGM/Mg2+ solid medium composed of yeast extract 5 g/L, glucose 20 g/L, mannitol 20 g/L, $MgSO_4$ 0.5 g/L, agar 15 g/L, pure ethanol 2% (v/v) and acetic acid 0.5 % (v/v) (Lisdiyanti et al., 2000). Ethanol and acetic acid were added to the medium aseptically after sterilization. Glucose and mannitol were sterilized separately. Cycloheximide and nisin were used in culture media to inhibit the growth of fungi and lactic acid bacteria, respectively (Kadere et al., 2008). The screening procedure was completed in Carr, Frateur and GYC solid media on plates at 30°C (Sievers and Swings, 2005). The isolated and purified strains were stored on GYC agar at 4ºC for a few days and

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in Microbank vials at -20ºC for long-term storage.

Identification of selected strains

Biochemical and morphological identification tests were performed to confirm that the selected isolates belong to Acetobacter genius. Conventional biochemical tests, including gram staining, catalase, oxidase, and oxidation of ethanol, were performed following the guidelines of Bergey's Manual of Systematic Bacteriology (Sievers and Swings, 2005). Other biochemical tests such as growth in the presence of high glucose concentrations, ammonium utilization and different carbohydrate assimilation were performed on presumed Acetobacter strains.

In addition, presumed Acetobacter strains were submitted to 16s rRNA sequence analysis by amplification of genomic DNA with universal primers 16SP0 5'-GAAGAGTTTGATCCTGGCTCAG-3' for the coding segment and 16SP6 5'-CTACGGCTACCTTGTTACGA-3' for the non-coding segment (Mounir et al., 2016). DNA was extracted from fresh cells grown on solid YGM/Mg2+ medium using the Promega extraction kit (Promega, USA). Then, the PCR reaction was monitored in a 200 µl Eppendorf tube containing 25 µl of Ready Mix (Promega, USA). The conditions of the PCR reaction, carried out in a thermocyclor (Eppendorf, France) were as follows: The first denaturation cycle of the DNA at 95°C for 5 min, 25 denaturation cycles at 95°C for 30 s, the primers annealing at 55°C for 30 s, the primers elongation at 72°C for 2 min, and a final elongation cycle at 72°C for 10 min. Thereafter, the PCR products were separated and visualized using electrophoresis at 100V for 20 min on 1% agarose gel in a phosphate TAE buffer 50x containing 1 µg/ml of ethidium bromide. The PCR reaction products were purified using the kit PCR Preps Wizard (Promega, USA) and quantified on agarose gel.

Finally, the purified PCR product was sequenced according to the Sanger method using a Big Dye Kit and a 3730 DNA analyzer (Applied Biosystems) (Ndoye et al., 2006), and the CodonCode Aligner program was used to pile up the products of the sequencing reaction. The sequences were then compared to those deposited in GenBank, using the BLAST algorithm (NCBI).

Analytical methods

Acetic acid was determined by titration using 0.5 M sodium hydroxide and phenolphthalein as indicator. This rapid method was used for immediate monitoring of acid production during fermentation. Biomass was estimated by optical density (O.D) measurement at 540 nm. Calibration curve was established between O.D and bacterial dried weight (g/L) determined using gravimetrical method (Chen et al., 2011; Ndoye et al., 2007c). Samples were passed through cellulose nitrate membrane (0.45 µm pore size) using a vacuum system. The membrane was then dried (105°C) until a constant weight.

High performance liquid chromatography (HPLC) was used to determine glucose, ethanol, acetate, and gluconate in cultures. Culture samples were collected and centrifuged at 13,000 rpm for 10 min and the supernatants were filtered through a 0.2 µm cellulose acetate membrane (Sartorius Minisart). The HPLC analyses were performed using an Agilent 1110 series HPLC equipped with a Supelcogel C610H column preceded by a Supelguard H precolumn (using a column heater at 40°C) and a differential refractive index detector (RID, detection cell maintained at 35°C). An isocratic mobile phase consisting of 0.1% H_3PO_4 (in MilliQ water) was used at a flow rate of 0.5 mL/min. Analysis was completed within 35 min and operated at a maximum pressure of 60 bars.

The bioconversion capacity of selected bacteria was determined by calculating the following parameters:

1. Stoichiometric yield:

$$Ys = \frac{n_f(AA) - n_i(AA)}{n_i(Eth) - n_f(Eth)} \text{ x } 100$$

n_i(AA) and n_i(Eth): initial acetic acid and ethanol moles, respectively. n_f(AA) and n_f(Eth): Final acetic acid and ethanol moles, respectively.

2. Theoretical acetic acid concentration (C_t): Considering the reaction

$C_2H_5OH + O_2 \rightarrow CH_3COOH + H_2O$

The theoretical acetic acid concentration C_t expressing the mass (g) of acetic acid formed in 100 ml was calculated as follows:

$$C_t = \frac{C_i\ (Eth) - C_f\ (Eth)}{M\ (Eth)}\ x\ M\ (AA) + C_0$$

C_i (Eth): initial ethanol concentration. C_f (Eth): final ethanol concentration. M (Eth): ethanol molar mass. M (AA): acetic acid molar mass. C_0: starting acetic acid concentration in the medium.

3. Bioconversion efficiency (E):

$E = (C_r / C_t) \times 100$

C_r: Real acetic acid concentration determined by titration. C_t: theoretical acetic acid concentration.

4. Productivity (P): quantity of acetic acid produced per liter and per hour (g/L/h).

Evaluation of bioconversion ability of the selected bacteria on fruit musts

In this part of the study, the ability of selected strains to achieve efficient acetic fermentation on alcoholic fermented fruit juices was evaluated in order to select the most efficient strains with regard to acetic acid and biomass production. Alcoholic musts used to perform acetic fermentation were obtained from date and apple juices. These fruits were chosen on the basis of their availability on the local market and on their significant valorization potential.

Fruit juice extraction

Date juice was prepared from black Bousthami date variety (south-east of Morocco). This variety was chosen because of its availability and its low cost. The soft consistency and sugar content of this variety give it very interesting properties to be transformed; it is widely used in the most popular, traditional preparations of soft dates in the south of Morocco (Harrak et al., 2012). The preparation of date juice was performed according to the soaking method recommended by Nancib et al. (2001), except that the temperature used for the extraction was 65°C for 2 h. The mixture was then filtered through a filter cloth to obtain clear juice. Apple juice was extracted using a Robot-Coupe centrifuge (J 80 Ultra, France) by pressing mature, small size apples. Date and apple juices were used immediately for fermentation.

Alcoholic fermentation

Alcoholic fermentation was achieved using a commercial baker's *Saccharomyces cerevisiae* "Rafiaa" strain. The yeast was obtained

Table 1. Coded levels (between brackets) and corresponding real levels of the independent variables involved in the design.

Variable		Levels				
		(-2)	(-1)	(0)	(1)	(2)
X1	Ethanol (g/L)	10	20	30	40	50
X2	Acetic acid (g/L)	6	8	10	12	14
X3	Glucose (g/L)	10	12.5	15	17.5	20
X4	pH	4	4.5	5	5.5	6

from LESAFFRE Company (Morocco) in the form of active dried granules. pH of date juice, which exceeded the optimum value of the yeast, was adjusted to 3.5 by adding 0.5 N citric acid (Colin and Conroy, 1998). The yeast was activated by mixing the appropriate amount (0.6 g/L of inoculated juice) with 500 ml of warm juice (35 to 40°C). After a rest period of 15 min, active yeast was used to inoculate a 30 L plastic drum of both apple and date juices.

Acetous fermentation

Prior to main fermentation, strains were screened using an enrichment medium composed of glucose 2% (w/v), yeast extract 1% (w/v), peptone of casein 0.5% (w/v), mannitol 2% (w/v), ethanol 2% (v/v) and acetic acid 1% (v/v). 500 ml flasks containing preculture media were incubated at 30°C on a shaker (120 rpm) for 2 days. The alcohol concentration of fruit musts was adjusted to 4% (v/v) which is the optimum for cell growth according to results reported by Kommanee et al. (2012) and Romero-Cortes et al. (2012). Afterwards, selected strains were evaluated for their ability to perform acetous fermentation of alcoholic fruit musts.

Optimization of biomass production of selected bacteria

Experimental design

In this part of the study, we investigated the effect of glucose, pH, acetic acid, and ethanol on the biomass production of selected bacteria using response surface methodology (RSM). The fermentation cultures were carried out in Erlenmeyer flasks (250 mL) containing 100 mL GYEA culture medium supplemented by 2.5 g/L fructose. Glucose, ethanol, acetic acid, and pH were then fixed based on the experimental design. Table 1 summarizes the independent variables involved in the design in their real and coded levels. Minimum and maximum levels of influencing variables studied were: 10 to 50 (g/L) for ethanol, 6 to 14 (g/L) for acetic acid, 10 to 20 (g/L) for glucose and 4 to 6 for pH. The lower and upper limits were determined with reference to previous experiments (data not shown). Accordingly, these conditions generated an experimental design (Table 5) with 30 runs determined by: 2^k=16 factorial points, 2k=8 axial points, and N_0=6 central points designed as replications.

By designating cellular biomass as "Y", the quadratic polynomial equation 1 which describes the variation of the response "Y" is as follows:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j + \varepsilon \quad (1)$$

Where β_0, β_i, β_{ii}, β_{ij} are constant regression coefficients of the model and X_i , X_j (i=1-4; j=1-4; i ≠ j) represent the independent variables. The coefficient of determination R^2 was used to express the quality of fit of the polynomial model. The experiments were carried out in duplicates and the mean values were calculated.

The *Acetobacter* strain selected for its best performance in terms of acetic acid production on alcoholic juices (apple and date) was chosen to maximize growth depending on the composition of the fermentation medium.

Statistical analysis

Central composite design was used to predict bacterial biomass production (Table 5). Data gained from the experiments were subjected to a second order multiple regression analysis to obtain parameters estimated for the mathematical model. Statistical analysis [regression and analysis of variance (ANOVA)] was carried out using Minitab software (v. 17 .1.0, UK, 2003). The contour plot and the 3D response surface analysis were made by keeping two independent variables at constant level and changing the other two independent variables, and then calculating the response "Y".

Batch fermentation in a bioreactor

The selected strain for which the optimization of biomass production was carried out was tested in 6-L scaled bioreactor (INFORS, France). The influencing parameters studied were taken in their statistically predicted optimal values to prepare the cultivation medium. The reactor was aerated using a continuous flow of filtered sterile air at a rate of 1 VVM. Microbial cells were first precultured in 500 ml baffled flask at 30°C for 36 h and then the liquid broth was inoculated into a fermenter. Bacterial growth was monitored by measuring dry matter (g/L) along with the produced acetic acid and residual ethanol.

RESULTS AND DISCUSSION

Identification of isolated bacteria

The present study aimed at isolating AAB strains from local Moroccan products destined for bacterial starter culture production to use in industrial vinegar production. The incubation of the inoculated culture media made of apple, cactus and date juice extracts at 30°C for 7 days resulted in an increase in the growth of presumptive acetic acid bacteria in the fermentation media. This was confirmed by the increase in the turbidity of the culture media and the development of the characteristic odor of acetic acid. Except for isolates obtained from figs, strains obtained from apple and date fruits (AF and DF), cactus vinegar (CV), and apple vinegar (AV) were able to convert Carr medium color from blue to yellow and could develop colonies surrounded by bright rings in Frateur and GYC media. These macroscopic observations show that the four groups of isolates were capable of converting ethanol present in the Carr medium in acetic acid, which resulted in a color change (Mounir et al., 2015; Sharafi et al., 2010).

According to biochemical tests (Table 2), isolated bacteria were gram-negative, catalase-positive, and oxidase-negative. Morphologically they appeared on GYC agar as smooth colonies in single or paired cocci and sometimes rod-shaped. A motility test made on a mannitol

Table 2. Biochemical and morphological identification of selected bacteria.

Test	Isolates [a]												
	***A. Aceti* (LMG1625)**	**FF1 G1 [b]**	**FF2 G2**	**AF1 G3**	**AF2 G4**	**CF1 G5**	**DF1 G6**	**DF2 G7**	**CV1 G8**	**CV2 G9**	**AV1 G10**	**AV2 G11**	**AV3 G12**
Morphology	Rods	Irregular	Irregular	Ovoid	Spherical	Ovoid	Ovoid	Ovoid	Ovoid	Spherical	Ovoid	Ovoid	Spherical
Arrangement	Pair	Single	Single	Pair/ single	Pair/ single	Pair/ single	Pair/ single	Pair/ single	Pair/ single	Pair/ single	Pair/ single	Pair/ single	Pair/ single
Motility	+	-	-	+	+	+	+	+	+	+	+	+	+
Gram staining	-	-	+	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-
Production of acetate from ethanol	+	-	-	+	+	+	+	+	+	+	+	+	+
Over-oxidation capacity	+	-	-	+	+	+	+	+	+	+	+	+	+
Cellulose production	-	-	-	-	-	-	-	-	-	-	-	**+**	-

[a]FF, fig fruit; AF, apple fruit; CF, cactus fruit; DF, date fruit; CV, cactus vinegar; AV, apple vinegar; [b]G1 - G12, groups of bacteria respectively formed of a number of 6, 4, 3, 7, 4, 5, 4, 8, 5, 6, 7 and 4 isolates.

nitrate motility medium showed that all selected bacteria were found to be motile. In addition to these results, molecular identification was performed according to phylogenetic analysis based on the sequencing of the gene coding for 16S ribosomal RNA. According to NCBI blast algorithm, the representative selected strains, which were isolated from apple fruit (AF), cactus vinegar (CV), and cactus fruit (CF), respectively, were closely related to *A. pasteurianus* [97-99% homology (query cover)], whereas the selected isolate from date fruit (DF) was related to *A. pomorum* with 98% homology.

Interestingly, the cultures of a selected strain, isolated from traditional apple vinegar (AV2 group), exhibited the formation of a non-soluble substance, which accumulated in the form of filaments. After identification with Lugol's solution (1/10) and Congo red, this substance was recognized as cellulose. The 16s rDNA sequence analysis revealed that this bacterium was assigned to *Komagataeibacter xylinus* (98% homology). Further studies aimed at the optimization of the production of this substance could be considered. Therefore, this phenomenon represents a disadvantage for the use of this bacterium for industrial production of vinegar; it is, however, more suited to be involved in industrial production of cellulose (Fu et al., 2014; Jozala et al., 2015; Qureshi et al., 2013). The GenBank accession numbers for the 16s rRNA sequences of the two selected bacteria, CV01 and AF01, isolated from cactus and apple fruits are KU710511 and KU710512, respectively. These bacteria were selected for further studies.

Evaluation of bioconversion ability of the selected bacteria

This part of the study aimed to evaluate the ability of the selected *Acetobacter* strains to perform acetous fermentation on apple and date fruit musts (Table 3). The bioconversion capacity of tested strains was estimated by calculating fermentation performance indicators (stoichiometric yield, theoretical acetate production, bioconversion efficiency and productivity) as described in the material and methods section. The first stage alcoholic fermentation was achieved using a commercial *S. cerevisiae* strain and allowed to obtain a final ethanol concentration after 10 days of 8.24 and 9.12% (v/v), respectively, for apple and date alcoholic musts. This result corroborated the depletion of reducing and total sugars and, consequently, with °Bx variation which represents the percent of soluble dry matter by weight (grams per 100 milliliter of water) (Table 3). The alcoholic fermenting media were adjusted for their starting ethanol and acetic acid concentrations of 4 and 1% (v/v), respectively before inoculation with active cells taken in their exponential growth phase

Table 3. Chemical analysis of apple and date juices and musts used for the bioconversion assay.

Parameter		Fruit juice		Fruit musts	
		Apple Golden delicious	Date Black bousthammi	Apple Golden delicious	Date Black bousthammi
pH		3.86±0.06	5.85±0.02	ND	ND
°Bx	% (w/w)	13.30±0.23	15.76±0.11	4.06±0.07	5.12±0.10
Acidity	% (w/w)	0.64±0.13	0.36±0.09	0.79±0.11	0.81±0.17
Ethanol	% (v/v)	ND	ND	8.24±0.08	9.12±0.33
Ash content	% (w/w)	0.22±0.05	1.45±0.12	0.18±0.1	1.33±0.23
Reducing sugars	% (w/v)	8.2±0.97	5.8±0.65	Traces	Traces
Total sugars	% (w/v)	12.2±1.45	17.65±1.39	3.16±0.16	5.41±0.21

(10^6 to 10^7 CFU/mL).

Figure 1 shows that the four tested bacteria were able to produce acetic acid from ethanol in both date and apple fruit fermented juices. The overall acetate production process finished in 5 days in both cases. However, the kinetics of oxidation and the final acetic acid concentration differed from one strain to another. A maximum amount of acetic acid productions (42.5 and 36.5 g/L) were obtained for the CV01 *Acetobacter pasteurianus* strain, respectively, for apple and date acetous fermentation processes. This was confirmed by the calculation of performance indicators. Indeed, the results summarized in Table 4 show that the CV01 strain exhibited the highest productivity (P) level in the two experiments compared to the others (0.27 and 0.22 g/L/h for apple and date fermentation media, respectively). On the other hand, the stoichiometric yield, which represents the moles of acetic acid produced per mole of ethanol consumed in the liquid medium (Ndoye et al., 2007b), ranged from 76.96 to 97.81% for the overall strains. Except for the AV1 strain, the final produced amount of acetic acid and then the productivity (P) level were higher in the apple fermentation process compared to the date process. This allowed us to claim that the fermented apple juice was more suited for acetic fermentation compared with the fermented date juice. Considering these results, the CV01 and AF01 strains were selected for further studies.

Optimization of biomass production of CV01 *Acetobacter* strain

An RSM experiment was performed to evaluate the effect of four independent variables (ethanol, acetic acid, glucose, and pH) on biomass production (g/L) of the selected CV01 *Acetobacter* strain isolated from cactus vinegar. According to the generated experimental design, 30 experiments were implemented separately in 500 ml baffled flasks incubated on a shaker (120 rpm) at 30°C. Flasks were inoculated by fresh cells grown on a plate (24 to 48 h) and the bacterial biomass was estimated after 72 h using the gravimetrical method. The coded, actual, and predicted values of the independent variables and their responses are shown in Table 5. The bacterial biomass ranged from 1.563 (run number 18) to 2.251 (run number 26). The analysis of variance (*F*-test) results are given in Table 6. These results could be explored for evaluating the overall quality of the model. The given value of the coefficient of determination R^2 (0.968) implies that 96.80% of the sample variation of biomass concentration was attributed to the independent variables, and only 3.20% of the total variation of biomass cannot be explained by the model. This suggests that the accuracy and general predictive ability of the polynomial model was acceptable since the R^2 value was higher than 0.9 (Li et al., 2005). In addition, the observed values of Fisher (*F*-value) and the corresponding probability (*P*-value) of the model (respectively, 32.38 and <0.001) show that the model is highly significant.

The effects of each factor and their interactions on bacterial biomass were estimated through the regression analysis shown in Table 7. The corresponding *P* values of regression coefficients are used as a tool to verify the significance of each coefficient, which in turn may indicate patterns of interaction between the coefficients (Cui et al., 2006). Statistically, the smaller the *P*-values, the greater the significance of the corresponding coefficient (Liu et al., 2003). Results reported in Table 7 show that the regression coefficients of all the quadratic terms and two of the linear coefficients (β_1 and β_4) were significant at the 1% level; furthermore, two of the cross-products (β_1 x β_2 and β_1 x β_4) were also found to be significant at the 1% level. Taking into account only terms found to be significant, the fitted second order polynomial equation for the prediction of the biomass production (g/L) of CV01 *Acetobacter* strain is shown below:

$$\text{Biomass (g/L)} = 2.1647 - 0.0620X_1 + 0.0581X_4 - 0.1215X_1^2 - 0.0758X_2^2 - 0.0550X_3^2 - 0.0404X_4^2 - 0.0335X_1X_2 + 0.0307X_1X_4 \quad (2)$$

Ethanol (X_1) and acetic acid (X_2) had a negative effect on

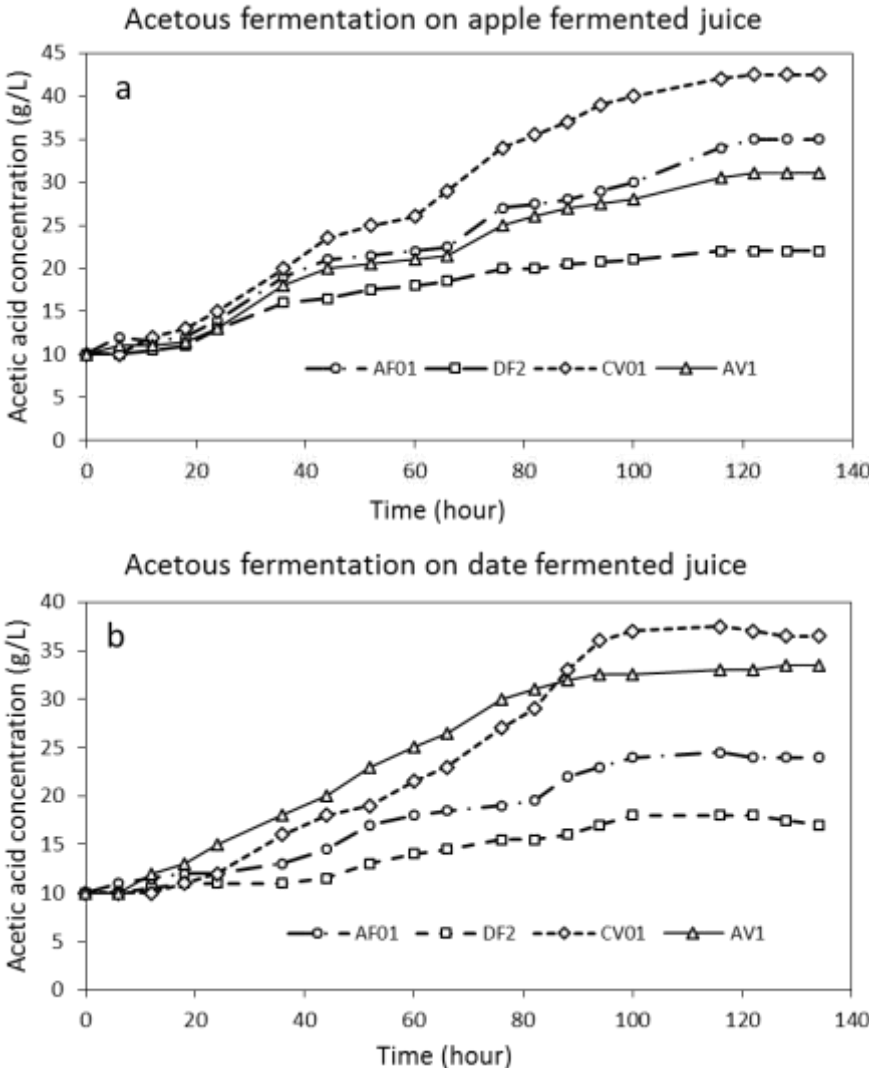


Figure 1. Acetous fermentation on apple (a) and date (b) fermented juices of four selected *Acetobacter* strains AF01, DF2, CV01 and AV1 isolated respectively from apple fruit, date fruit, cactus vinegar and apple vinegar. Fermentation was carried out in 500 ml flasks at 30°C.

Table 4. Calculation of performance indicators for selected strains subjected to acetous fermentation on fruit fermented juices.

Strain	AA_f[a] (% m/v)		Eth_f[b] (% v/v)		C_t[c] (% w/v)		Ys[d](%)		E[e] (%)		P[f] (g/L.h)	
	Apple	Date	Apple	Date	Apple	Date	Apple	Date	Apple	Date	Apple	Date
AF01	3.50	2.40	1.27	2.37	3.70	2.61	92.62	86.87	94.62	91.90	0.21	0.12
DF2	2.20	1.70	2.43	3.08	2.55	1.91	77.31	76.96	86.20	89.02	0.10	0.06
CV01	4.25	3.65	0.42	1.25	4.54	3.72	91.82	97.47	93.62	98.15	0.27	0.22
AV1	3.10	3.35	1.65	1.57	3.32	3.40	90.38	97.81	93.28	98.46	0.18	0.20

[a]Final acetic acid concentration; [b]final ethanol concentration (obtained at the end of acetous fermentation from fruit musts with starting ethanol concentration of 4% (v/v)); [c] theoretical acetic acid concentration; [d] stoichiometric yield; [e] bioconversion efficiency; [f] productivity.

cellular biomass, whereas glucose (X_3) and pH (X_4) had a positive effect. This finding agrees with several previous studies (Garrido-Vidal et al., 2003; González-Sáiz et al., 2009; Macías et al., 1997). In particular, Chen et al. (2011) reported the same effect of glucose and pH on biomass production of *Acetobacter* sp. CCTCC M209061

Table 5. Central composite design matrix and the responses of the dependent variable biomass.

Runs	Coded independent variable levels				Actual values of independent variables				Biomass (g/L)	
	X1	*X2*	*X3*	*X4*	Ethanol (g/L)	Acetic acid (g/L)	Glucose (g/L)	pH	Experimental	Predicted
1	-1	-1	-1	-1	40	8	12.5	4.5	1.887	1.902
2	1	-1	-1	-1	20	8	12.5	4.5	1.793	1.762
3	-1	1	-1	-1	20	12	12.5	4.5	1.912	1.915
4	1	1	-1	-1	40	12	12.5	4.5	1.633	1.641
5	-1	-1	1	-1	20	8	17.5	4.5	1.919	1.885
6	1	-1	1	-1	40	8	17.5	4.5	1.755	1.788
7	-1	1	1	-1	20	12	17.5	4.5	1.956	1.924
8	1	1	1	-1	40	12	17.5	4.5	1.702	1.694
9	-1	-1	-1	1	20	8	12.5	5.5	1.947	1.930
10	1	-1	-1	1	40	8	12.5	5.5	1.891	1.913
11	-1	1	-1	1	20	12	12.5	5.5	2.041	1.999
12	1	1	-1	1	40	12	12.5	5.5	1.839	1.847
13	-1	-1	1	1	20	8	17.5	5.5	1.929	1.911
14	1	-1	1	1	40	8	17.5	5.5	1.965	1.938
15	-1	1	1	1	20	12	17.5	5.5	2.000	2.006
16	1	1	1	1	40	12	17.5	5.5	1.923	1.898
17	-2	0	0	0	10	10	15	5	1.762	1.803
18	2	0	0	0	50	10	15	5	1.563	1.555
19	0	-2	0	0	30	6	15	5	1.865	1.875
20	0	2	0	0	30	14	15	5	1.825	1.848
21	0	0	-2	0	30	10	10	5	1.930	1.928
22	0	0	2	0	30	10	20	5	1.927	1.962
23	0	0	0	-2	30	10	15	4	1.883	1.887
24	0	0	0	2	30	10	15	6	2.091	2.119
25	0	0	0	0	30	10	15	5	2.163	2.165
26	0	0	0	0	30	10	15	5	2.251	2.165
27	0	0	0	0	30	10	15	5	2.126	2.165
28	0	0	0	0	30	10	15	5	2.145	2.165
29	0	0	0	0	30	10	15	5	2.182	2.165
30	0	0	0	0	30	10	15	5	2.124	2.165

Table 6. Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of CV01 biomass production.

Source	df	Sum of squares	Mean square	*F*-value	*P*-value
Model	14	0.745	0.053	32.38	<0.001**
Linear	4	0.176	0.044	26.77	<0.001**
Quadratic	4	0.530	0.132	80.66	<0.001**
Crossproduct	6	0.038	0.006	3.92	0.015*
Residual total error	15	0.025	0.002	-	-

**Significant at 1% level; * significant at 5% level; R^2 = 0.9680; Adj. R^2 = 0.9381.

grown on synthetic medium. The fitted model given in equation 1 indicates that ethanol concentration (X_1) had a significant linear effect (P<0.001) on cellular biomass of the studied strain as it has the higher coefficient followed by pH (X_4). Only the linear coefficient of pH (X_4) and the interaction term (X_1X_4) had a positive value, which indicates a direct effect on biomass production. In contrast, the linear coefficient of ethanol (X_1) along with the quadratic terms (X_1^2, X_2^2, X_3^2 and X_4^2) and the interaction term (X_1X_2) had a negative effect that

Table 7. Regression analysis of a polynomial model for optimization of biomass production of CV01 strain.

Term	Estimated coefficients	*t*- Statistic	*P*-value
Intercept (β_0)	2.1647	130.74	<0.001**
β_1	-0.0620	-7.49	<0.001**
β_2	-0.0066	-0.80	0.436
β_3	0.0085	1.02	0.323
β_4	0.0581	7.02	<0.001**
β_1^2	-0.1215	-15.68	<0.001**
β_2^2	-0.0758	-9.78	<0.001**
β_3^2	-0.0550	-7.10	<0.001**
β_4^2	-0.0404	-5.22	<0.001**
$\beta_1 \times \beta_2$	-0.0335	-3.30	0.005**
$\beta_1 \times \beta_3$	0.0108	1.07	0.303
$\beta_1 \times \beta_4$	0.0307	3.03	0.008**
$\beta_2 \times \beta_3$	0.0066	0.65	0.527
$\beta_2 \times \beta_4$	0.0139	1.37	0.191
$\beta_3 \times \beta_4$	-0.0004	-0.04	0.966

** Significant at 1% level.

decreases cellular biomass.

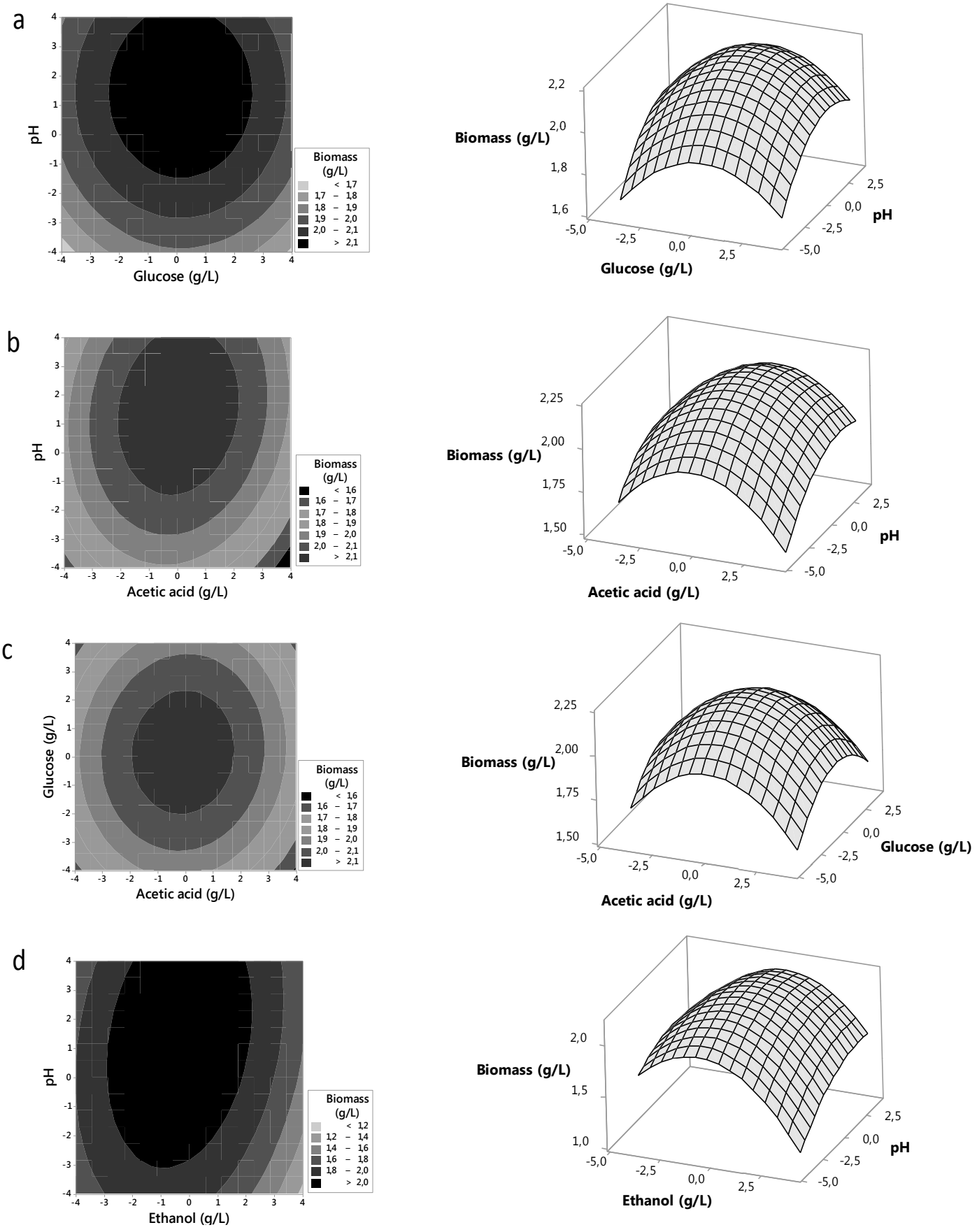
In order to better understand the relationship between the cellular biomass and the independent variables (X_1, X_2, X_3 and X_4), 3D response surface plots were formed based on the second order polynomial model. The shape of the corresponding contour plots indicates whether the mutual interactions between the independent variables are significant or not (Cui et al., 2006). Figure 2 (a-f) illustrates the fitted response surfaces and corresponding contour plots that provide a geometrical representation of changes in the predicted concentration cellular biomass, in response to modifications to two experimental parameters and maintaining the two others constant. Globally, variables exhibited a significant interaction. In fact, it is known that an elliptical contour plot indicates a significant interaction between variables (Liu et al., 2008). All six contour plots show similar relationships with respect to the effect of each variable. The three-dimensional plots and their respective contour plots facilitate the location of optimum experimental conditions (Liu et al., 2008). It is noteworthy that the produced biomass concentration of CV01 *Acetobacter* strain was sensitive when pH and ethanol concentration were subjected to a small alteration. The optimal values of variables required to obtain the maximum value of biomass concentration were gained by moving along the major and the minor axis of the contour plots. The predicted optimal values for the variables gained using the response optimizer command of the software were as follows: X_1= 28.18 g/L, X_2=10.12 g/L, X_3=15.15 g/L and X_4=5.33. The studied variables taken at these levels allow a production of a fit cellular biomass of approximately 2.21 g /L.

Batch fermentation in a 6-L bioreactor

A batch fermentation was performed for the CV01 strain in a 6 L Lab-fermenter (INFORS, France) in order to test the accuracy of the regression fitted model. The fermentation medium was prepared based on the predicted optimal composition. Thus, the medium was composed of 28.18 g/L ethanol, 10.12 g/L acetic acid, and 15.15 g/L glucose along with mannitol 20 g/L and fructose 2.5 g/L, and the pH was fixed at 5.33. Figure 3 shows the batch profile of CV01 *Acetobacter* strain in a lab-bioreactor and the variation of produced biomass, acetic acid and residual ethanol versus time. As can be seen in Figure 3, after a short adaptation time, the ethanol concentration started to decrease with a corresponding increase in acetic acid, and they reached the final concentration of 4.09 and 42.27 g/L for residual ethanol and acetic acid, respectively. On the other hand, bacterial biomass concentration increased slightly at the beginning of fermentation, then exponentially after 15 h. Bacterial biomass concentration reached a maximum level of 2.294 g/L after 80 h of fermentation. Consequently, the experimental value of produced biomass is shown to be slightly higher than the fit value (2.2 g/L). The raison might be related to the improvement of the volumetric oxygen transfer coefficient (K_La) in the bioreactor (better aeration and stirring systems) (De Ory et al., 2004).

Conclusions

The conversion of ethanol to acetic acid for the


a
pH
Glucose (g/L)
Biomass (g/L)
< 1,7
1,7 – 1,8
1,8 – 1,9
1,9 – 2,0
2,0 – 2,1
> 2,1
Biomass (g/L)
Glucose (g/L)
pH
b
pH
Acetic acid (g/L)
Biomass (g/L)
< 1,6
1,6 – 1,7
1,7 – 1,8
1,8 – 1,9
1,9 – 2,0
2,0 – 2,1
> 2,1
Biomass (g/L)
Acetic acid (g/L)
pH
c
Glucose (g/L)
Acetic acid (g/L)
Biomass (g/L)
< 1,6
1,6 – 1,7
1,7 – 1,8
1,8 – 1,9
1,9 – 2,0
2,0 – 2,1
> 2,1
Biomass (g/L)
Acetic acid (g/L)
Glucose (g/L)
d
pH
Ethanol (g/L)
Biomass (g/L)
< 1,2
1,2 – 1,4
1,4 – 1,6
1,6 – 1,8
1,8 – 2,0
> 2,0
Biomass (g/L)
Ethanol (g/L)
pH

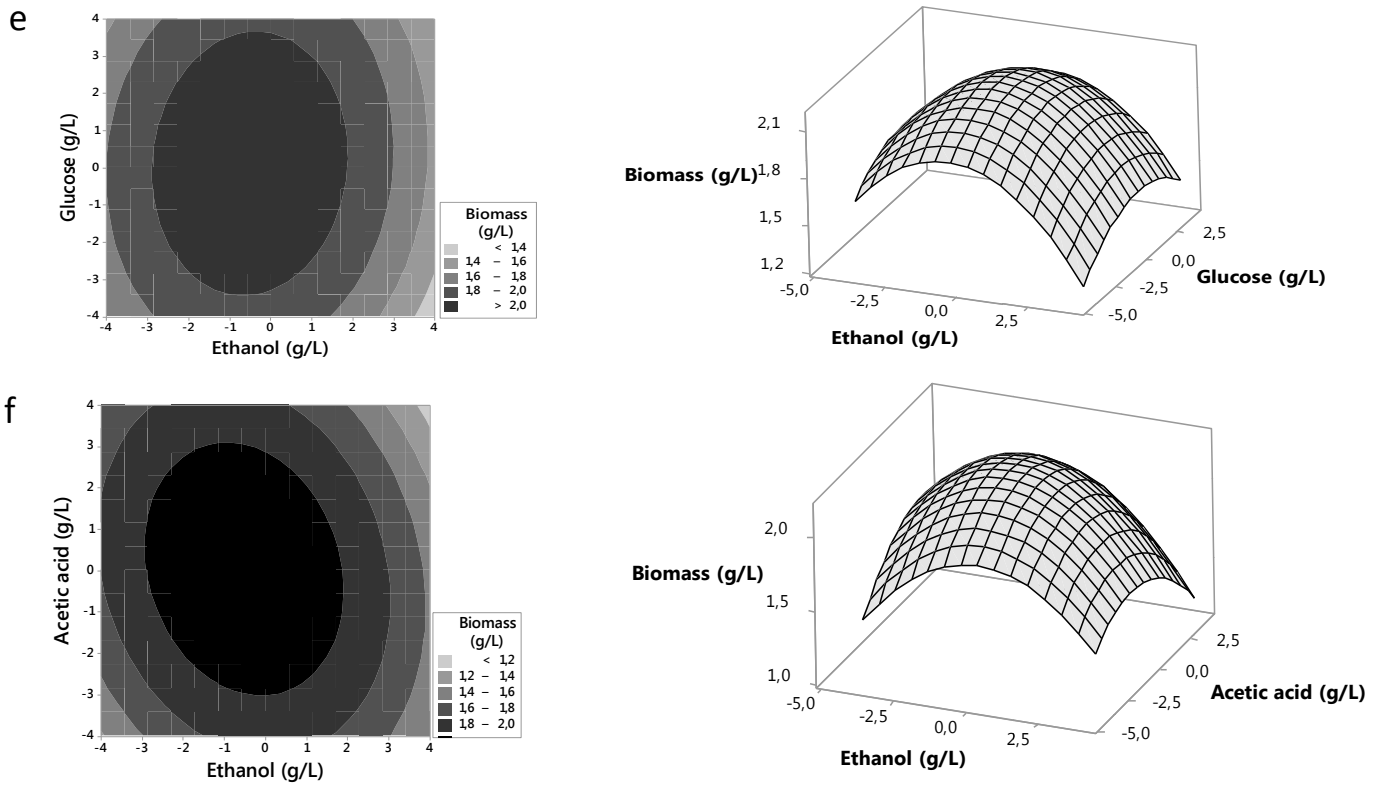


Figure 2. Surface plot and corresponding contour plot of the combined effects, respectively, of (a) glucose and pH; (b) acetic acid and pH; (c) acetic acid and glucose; (d) ethanol and pH; (e) ethanol and glucose, and (f) ethanol and acetic acid on the cellular biomass production of CV01 *Acetobacter* strain.

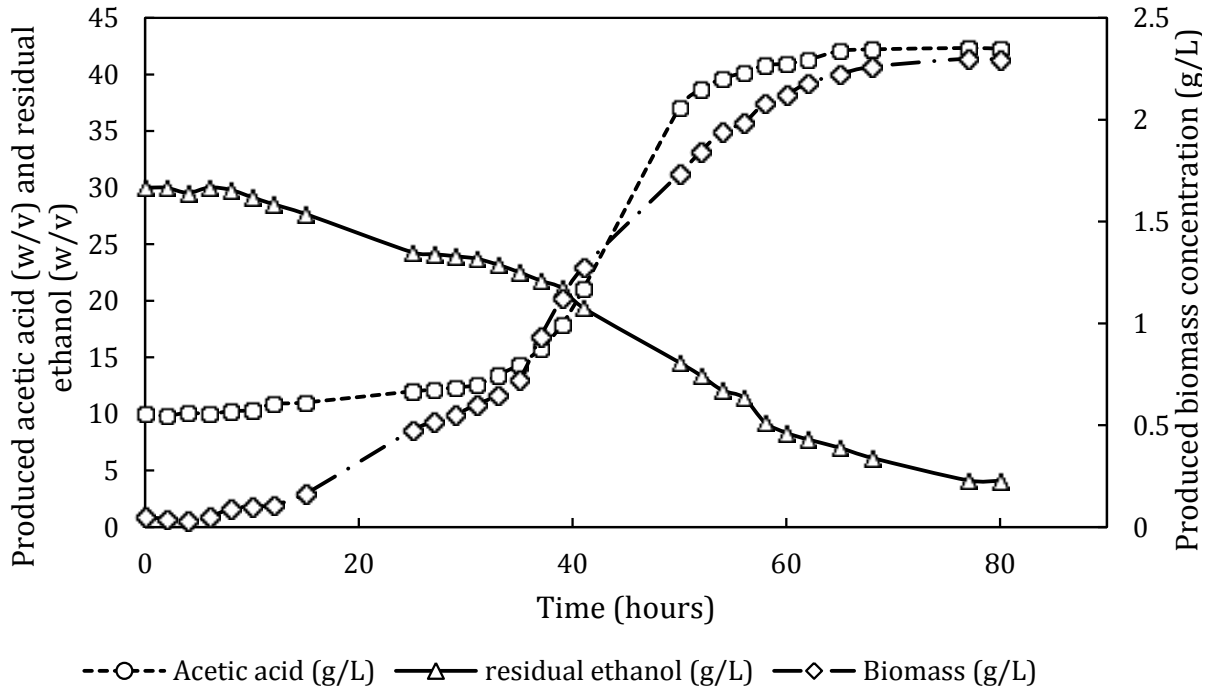


Figure 3. Batch fermentation profile of *Acetobacter* CV01 strain in a 6-L bioreactor at 30°C (a), and at thermal stress condition (b). Cultures were carried out using the optimized culture medium composition. The presented results are the means of two independent replicates.

production of vinegar is the most well-known application of AAB strains. In this paper, novel bacteria considered to be *Acetobacter* species according to a phylogenetic study based on 16s rDNA gene sequence analysis were isolated. These strains were subjected to an oxidation ability test performed on date and apple juices. Maximum acetic acid productions (42.5 and 36.5 g/L) were obtained for the CV01 *A. pasteurianus* strain isolated from cactus vinegar, respectively, for apple and date acetous fermentation processes. Response surface methodology (RSM) was applied to maximize the production of cell biomass of the CV01 *Acetobacter* strain for an industrial starter production objective. It was concluded that the predicted optimal values for the studied variables (ethanol, acetic acid, glucose and pH), allowing the maximum biomass production of 2.2 g/L, were, respectively, as follows: X_1= 28.18 g/L, X_2=10.12 g/L, X_3=15.15 g/ and X_4=5.33. Finally, a batch fermentation was carried out in a 6-L lab-bioreactor and the results were in line with the predicted values. It was concluded that the CV01 strain was well suited to be used as parental strain to prepare a starter for vinegar fruit production. Consequently, the conservation of this strain through freeze-dried powder lyophilisation is to be considered.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors wish to thank the BTC (Belgian Development Agency) for financial support (grant number 12MOR/0113) accorded to M. Mounir.

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