

Research Article

A Low Cost Medium Containing Chicken Intestine Autolysate for *Lactobacillus brevis* Growth: Statistical Optimization

¹Kelthoum Ahmed-Gaïd, ¹Messaouda Boukhemis and ²Baida Djeghri-Hocine

¹Laboratoire de Biochimie et de Microbiologie Appliquée, Département de Biochimie, Faculté des Sciences, Université de Badji Mokhtar BP12, Annaba, 23000,

²École Nationale Supérieure des Sciences de la Mer et de L'aménagement du Littoral, Alger, Algérie

Abstract: The valorization of chicken intestine to formulate a growth medium for lactic acid bacteria was tested on a strain of *Lactobacillus brevis* CHTD27. For this purpose, an autolytic degradation of the chicken intestine was carried out. The resulted Chicken Intestine Autolysate (CIA) was used as basic medium, while other medium parameters were studied using a Plackett and Burman design to determine the significant factors affecting the strain growth. Those were further optimized using a central composite design. As a result, CIA containing 19.15 g/L of glucose and 0.25 g/L of ammonium citrate supported good growth of *L. brevis* with a final optical density value of 6.00 at 600 nm, which is 32% higher than that on the standard medium MRS. Thus, medium containing CIA could constitute a low-cost alternative to MRS medium for the production of *L. brevis* biomass.

Keywords: Autolysis, chicken intestine, growth medium, *Lactobacillus brevis*, statistical optimization

INTRODUCTION

Actually, Lactic Acid Bacteria (LAB) are exploited in various areas for their beneficial properties. They are used as starter cultures for the elaboration of fermented food (LeBlanc *et al.*, 2010). They are also the major representatives of probiotics both on the food and the pharmaceutical markets (Franz *et al.*, 2010). Looking to their importance, LAB must be produced in large scales. A commonly used growth medium is Man, Rogosa, Sharpe medium (MRS), but this medium is too expensive because it is composed of costly nitrogen sources. For example, yeast extract, which is important for a high lactic acid production is estimated to account for about 38% of the total production cost (Hujanen *et al.*, 2001). To reduce the cost of LAB biomass production, several studies undertook the formulation of low-cost media for LAB using low-cost substrates or by-products generally known to be rich in protein (Aspmo *et al.*, 2005; Horn *et al.*, 2005; Safari *et al.*, 2009).

In Algeria, because of the important consumption of poultry meat, poultry slaughterhouses generate substantial amounts of by-products every day and chicken intestine is one of the major wastes from this industry. Indeed, slaughterhouses wastes including poultry by-products are not valorised in Algeria, but are usually buried or incinerated and may consequently lead to environmental contamination and pose a threat

to animal and human health (Escudero *et al.*, 2014). Therefore, it is important to find alternative solutions for their treatment (Edström *et al.*, 2003).

In order to solve the problems of high amounts of animal's by-products and LAB biomass production costs, this study proposes, as a solution, the exploitation of chicken intestines to formulate a growth medium for a LAB strain (*Lactobacillus brevis*). The use of statistical experimental designs allowed to define easily and exactly the composition of the new growth medium. The growth of *L. brevis* on the optimized medium was compared with that on the commercial medium (MRS) to define which one supported the highest growth of the strain.

MATERIALS AND METHODS

Preparation of Chicken Intestine Autolysate (CIA): Chicken intestines were collected from the slaughterhouse of DraaErrich in Annaba, Algeria immediately after the slaughtering. They were thoroughly washed in running water to remove their content and frozen in boxes at -20°C until use. The autolytic degradation was carried out according to Jamdar and Harikumar (2008); a homogenate composed of 40% of chicken intestine and 60% of distilled water was ground in a Moulinex blender for 5 min. pH was adjusted to 2.8 by adding 2N HCl. After that, the homogenate was dispensed into 250-mL Erlenmeyer

Corresponding Author: Kelthoum Ahmed-Gaïd, Laboratoire de Biochimie et de Microbiologie Appliquée, Département de Biochimie, Faculté des Sciences, Université de Badji Mokhtar BP12, Annaba, 23000, Algérie, Tel.: +213 797 17 81 87; Fax: +213 38 87 26 53

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flasks and incubated in a shaking incubator at 55°C and 150 rpm for 6 h. Autolysis was terminated by inactivation of samples at 90°C for 20 min; after heating, the clear supernatant phase was recuperated, centrifuged at 4000 g for 25 min and filtered to obtain the CIA.

Proximate composition: The protein concentration was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard, total sugars were determined according to Dubois *et al.* (1956) and lipid content according to Folch *et al.* (1957). Dry matter was calculated after drying samples at 105°C for 24 h, ash content after heating to 550°C for 5 h.

Microorganism and bacterial growth estimation: *Lactobacillus brevis* CHTD27 from the collection of Laboratoire de Biologie des Microorganismes et Biotechnologie (LBMB) (Essenia, Oran, Algeria) was originally isolated from raw camel milk collected in *Tindouf* (Roudj *et al.*, 2009). The strain was stored as frozen stock held at -20°C in milk, monthly reactivated in sterile MRS broth and propagated on MRS agar slants at 37°C. After fermentation, the optical density of samples was measured at 600 nm (OD₆₀₀) to estimate the biomass concentrations. Samples were diluted with their corresponding media to ensure that the OD₆₀₀ value is less than 0.6.

Medium and growth conditions: Cultures were carried out in flasks of 200 ml containing 30 mL of media composed of CIA as a basic medium. Other components, pH and agitation speed were varied according to the experimental design. After autoclaving and cooling, media were inoculated with 3% of the inoculum and incubated in a rotary shaker at 37°C for 24 h.

Experimental designs:

Plackett and Burman Design (PBD):

PBD is a fractional factorial design used to select, among a large number of factors, those having the most important effect on the studied response. It is a useful tool because it allows the study of *k* number of factors

in only *k*+1 runs (Plackett and Burman, 1946). The model is of the form:

$$\hat{Y} = \beta_0 + \sum \beta_i X_i$$

where,

- \hat{Y} = The response (OD₆₀₀)
- β_0 = The constant
- β_i = The coefficients of linear terms
- X_i = The coded forms of variables

In this study, PBD was used to evaluate the effects of medium parameters on *L. brevis* growth. The total number of experiments was 12 (Table 1). The variables were medium components (Glucose, yeast extract, ammonium citrate, K₂HPO₄, MgSO₄, MnSO₄ and Tween 80), physicochemical parameters (pH and agitation) and tow dummy (*X*₅ and *X*₁₁). Variables were tested at two levels: higher and lower (Table 2). The statistical significance of the regression coefficients was determined by Student's t-test using statistical software package MINITAB version 16.2.1.0. Variables showing a confidence level greater than 95% were considered to significantly influence the strain growth.

Central Composite Design (CCD): To maximize the growth of *L. brevis*, variables selected by the PBD to have significant effects were optimized using CCD. Factors were tested at five levels shown in Table 3. The second order polynomial equation is the following:

$$\hat{Y} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_{12} + \beta_{22} X_{22} + \beta_{12} X_1 X_2$$

where,

- \hat{Y} = The response
- β_0 = The model constant
- X_1 and X_2 = The independent variables
- β_1 and β_2 = The linear coefficients
- β_{11} and β_{22} = The quadratic coefficients
- β_{12} = The cross product coefficient

CCD results were interpreted using MINITAB software version 16.2.1.0. The significance of the model was checked by the R² value and the model *F*-value. The 3D surface plot was realized using Design

Table 1: Plackett-Burman experimental design matrix and OD₆₀₀ results

| Run order | Factors | | | | | | | | | | | OD ₆₀₀ |
|-----------|----------------|----------------|----------------|----------------|-------------------|----------------|----------------|----------------|----------------|-----------------|--------------------|-------------------|
| | X ₁ | X ₂ | X ₃ | X ₄ | (X ₅) | X ₆ | X ₇ | X ₈ | X ₉ | X ₁₀ | (X ₁₁) | |
| 1 | +1 | +1 | -1 | +1 | +1 | +1 | -1 | -1 | -1 | +1 | -1 | 1.5780 |
| 2 | -1 | +1 | +1 | -1 | +1 | +1 | +1 | -1 | -1 | -1 | +1 | 1.0580 |
| 3 | +1 | -1 | +1 | +1 | -1 | +1 | +1 | +1 | -1 | -1 | -1 | 4.7316 |
| 4 | -1 | +1 | -1 | +1 | +1 | -1 | +1 | +1 | +1 | -1 | -1 | 0.3740 |
| 5 | -1 | -1 | +1 | -1 | +1 | +1 | -1 | +1 | +1 | +1 | -1 | 1.6000 |
| 6 | -1 | -1 | -1 | +1 | -1 | +1 | +1 | -1 | +1 | +1 | +1 | 0.4430 |
| 7 | +1 | -1 | -1 | -1 | +1 | -1 | +1 | +1 | -1 | +1 | +1 | 3.1660 |
| 8 | +1 | +1 | -1 | -1 | -1 | +1 | -1 | +1 | +1 | -1 | +1 | 2.8530 |
| 9 | +1 | +1 | +1 | -1 | -1 | -1 | +1 | -1 | +1 | +1 | -1 | 3.1750 |
| 10 | -1 | +1 | +1 | +1 | -1 | -1 | -1 | +1 | -1 | +1 | +1 | 1.0420 |
| 11 | +1 | -1 | +1 | +1 | +1 | -1 | -1 | -1 | +1 | -1 | +1 | 4.0980 |
| 12 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 0.3300 |

+1: Higher level. -1: Lower level. X₅ and X₁₁ are Dummies

Table 2: Levels of the variables tested in Plackett–Burman design

| Factors | Symbol | High level (-1) | Low level (+1) |
|---------------------------|----------|-----------------|----------------|
| Glucose (g/L) | X_1 | 0 | 20 |
| Yeast extract (g/L) | X_2 | 0 | 5 |
| Ammonium citrate (g/L) | X_3 | 0 | 2 |
| Potassium phosphate (g/L) | X_4 | 0 | 2 |
| Dummy | X_5 | - | - |
| Magnesium sulfate (g/L) | X_6 | 0 | 0.2 |
| Manganese sulfate (g/L) | X_7 | 0 | 0.05 |
| Tween 80 (mL) | X_8 | 0 | 1 |
| pH | X_9 | 5.8 | 6.5 |
| Agitation (rpm) | X_{10} | 0 | 150 |
| Dummy | X_{11} | - | - |

Table 3: Central composite design for testing two factors: glucose and ammonium citrate

| Factors | Levels | | | | |
|------------------|----------|-----|------|-----|----------|
| | -1,41421 | -1 | 0 | +1 | +1,41421 |
| Glucose | 9.307 | 12 | 18.5 | 25 | 27.692 |
| Ammonium citrate | 0.017 | 0.1 | 0.3 | 0.5 | 0.582 |

Expert version 9.0.3.1 (STAT-EASE Inc., Minneapolis, MN, USA).

Validation experiments: The confirmation test was carried out in triplicate by the cultivation of the strain in CIA supplemented with the optimized concentrations of factors obtained by CCD. The observed and the predicted values of OD₆₀₀ were compared to judge the model validity.

Comparison of *L. brevis* growth on CIA medium and MRS medium: To evaluate the efficiency of the CIA as a growth medium, the strain was cultivated for 24 h in MRS medium and the optimized medium, then, final OD₆₀₀ values obtained were determined and compared.

RESULTS AND DISCUSSION

Proximate composition: Dry matter in the CIA was estimated at 0.5%. DM chemical composition was

87.78% of protein, 1.18% of fat, 0.26% of sugar and 9.5% of ash. Protein content was close to that found by Jamdar and Harikumar (2008) in chicken viscera autolysate obtained using the same autolysis degradation process (content of 84%). The high protein content revealed the efficiency of the autolytic degradation to produce good quality of hydrolyzate; this is mainly due to the activity of endogenous aspartic proteases present in intestinal tissue (Jamdar and Harikumar, 2005).

Screening of significant parameters influencing the cell growth: PBD was used to screen the influence of nine variables on the growth of *L. brevis*, OD₆₀₀ results are shown in Table 1, the significance of each variable checked by Student “t” test is shown in Table 4. Analysis of PBD results showed different levels of influence; the value of R² was 99.59%, while the adjusted R² was 97.74%.

Figure 1 represents Pareto chart graph and the *p*-line that distinguishes the significant factors and bar lengths proportional to the factors effects. Glucose (X_1), yeast extract (X_2) and ammonium citrate (X_3) were found to exert a significant effect on biomass production (\hat{Y}) with *p*-values of 0.003, 0.032 and 0.01 respectively. The model equation for biomass concentration was expressed in terms as following:

$$\hat{Y} = 2,037 + 1,229 X_1 - 0,357 X_2 + 0,580 X_3$$

Other variables showed *p*-values higher than 0.05 and considered as not significant. It was clear that glucose had the most important effect on *L. brevis* growth. This may be explained by the poorness of CIA in sugars and the necessity of a carbon source for the growth, since the critical ingredient in MRS medium

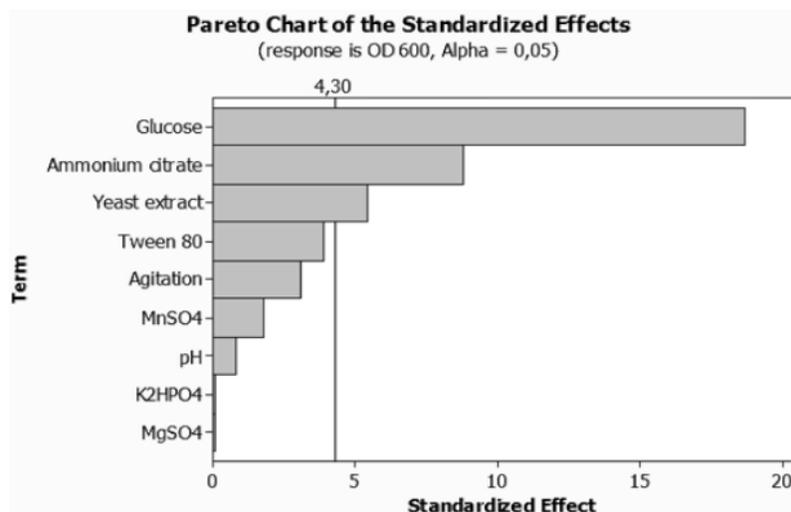


Fig. 1: Pareto chart for screening of significant effects on *L. brevis* CHTD27 growth

Table 4: Statistical analysis of Plackett-Burman design results

| Term | Effect | Coef. | SE Coef. | t-value | p-value |
|---------------------------------|---------|---------|----------|---------|---------|
| Constant | | 2.0374 | 0.06589 | 30.92 | 0.001 |
| Glucose* | 2.4591 | 1.2295 | 0.06589 | 18.66 | 0.003 |
| Yeast extract* | -0.7148 | -0.3574 | 0.06589 | -5.42 | 0.032 |
| Ammonium citrate* | 1.1601 | 0.5801 | 0.06589 | 8.80 | 0.013 |
| K ₂ HPO ₄ | 0.0141 | 0.0070 | 0.06589 | 0.11 | 0.925 |
| MgSO ₄ | 0.0131 | 0.0066 | 0.06589 | 0.10 | 0.930 |
| MnSO ₄ | 0.2411 | 0.1206 | 0.06589 | 1.83 | 0.209 |
| Tween 80 | 0.5141 | 0.2571 | 0.06589 | 3.90 | 0.060 |
| pH | 0.1062 | 0.0531 | 0.06589 | 0.81 | 0.505 |
| Agitation | -0.4068 | -0.2034 | 0.06589 | -3.09 | 0.091 |

R² = 99.59%, R²(pred) = 85.18%, R²(adj) = 97.74%; *: Statistically significant at 95% confidence level

Table 5: Analysis of variance for the quadratic model

| | Coef | SE Coef | t- value | p- value |
|---------------------------------|----------|---------|----------|----------|
| Constant | 6.06600 | 0.02741 | 221.294 | 0.000 |
| X ₁ | 0.09696 | 0.02167 | 4.474 | 0.003 |
| X ₂ | -0.23189 | 0.02167 | -10.701 | 0.000 |
| X ₁ ² | -0.46487 | 0.02324 | -20.004 | 0.000 |
| X ₂ ² | -0.48488 | 0.02324 | -20.865 | 0.000 |
| X ₁ . X ₂ | 0.00250 | 0.03065 | 0.082 | 0.937 |

X₁ is glucose and X₂ is ammonium citrate; R² = 99.21%, R²(pred) = 95.31%, R²(adj) = 98.64%

for *Lactobacillus* are those related to the carbon source concentration (Zhang *et al.*, 2014). Nitrogen sources present in the medium were CIA, yeast extract (organic nitrogen sources) and ammonium citrate (inorganic nitrogen source). From the PBD results, yeast extract and ammonium citrate were selected as significant factors, however ammonium citrate had a positive effect while yeast extract influenced negatively the cell growth. The suitable explanation of these results is that inorganic nitrogen sources are quickly metabolized by microorganisms for fast growth, while organic sources are sustainable nitrogen sources (Baixin *et al.*, 2011). On another hand, Vazquez *et al.* (2004) studied the use of fish viscera autohydrolysates for LAB growth and supposed that LAB could maintain a stable consortium in the intestinal space because of the availability of peptidic fractions produced by proteolytic enzymes of the digestive tract. They concluded that the autohydrolysis of viscera homogenates could generate peptidic fragments (peptones) suitable for the culture of lactic acid bacteria. Regarding the fact that LAB exist naturally in chicken intestine and its autolysis was carried out using its own proteolytic enzymes (autolytic degradation), the hypothesis of Vazquez *et al.* (2004) describes exactly our case. So we can suggest that CIA may contain peptidic fragments suitable for the LAB strain growth. Thereby, yeast extract, as an organic nitrogen source seemed efficiently replaced by CIA and its addition in the medium appears to be undesirable and justifies its negative effect. Concerning nitrogen sources, we suppose that *L. brevis* used ammonium citrate and CIA which recovered sufficiently its requirements, while yeast extract was not needed and might be a superfluous ingredient which causes growth inhibition when added.

Tween 80 was found to have an insignificant effect; this result corroborate that obtained by Gao *et al.* (2009) who indicated that Tween 80 is not required by *L. fermentum*. K₂HPO₄ effect was insignificant, similarly, Zhang *et al.* (2014) indicated that K₂HPO₄ had no significant effect on the growth of *L. rhamnosus*. MgSO₄ and MnSO₄ showed no significant influence on the growth. The insignificance of pH influence is in accordance with the result found by Aasen *et al.* (2000) who showed that pH effect on *L. sakei* growth is relatively small and the optimum pH for its growth is 6-6.5. The agitation effect was insignificant; Brinques *et al.* (2010) also observed this result in a study undertaken *L. plantarum* biomass production.

Eventually, positive significant variables (glucose and ammonium citrate) were included in the central composite experiment for the optimization. For the next experiments, the pH of media was adjusted to 6.5 and the cultures were carried out in an incubator without agitation.

Optimization of significant variables: CCD was used to optimize the concentrations of two factors selected by the PBD (glucose and ammonium citrate). Analysis of variance ANOVA of the results is shown in Table 5. The large variation of OD₆₀₀ values ranged from 4.80 to 6.09 and reflected the necessity of the medium optimization to attain optimal growth potentials. The coefficient of determination R² was 0.9921 which is so close to 1; this indicates the ability of the model to explain 99.21% of data variability and suggests an excellent correlation between observed and predicted OD₆₀₀ values. The adjusted R² value of 98.64 % indicates that the model is significant. The lack of fit was found to be insignificant (0.082) and the high *F*-value (174.74) support the high significance of the model. *p*-value of the model was less than 0.001 and for the two experimented factors, linear and quadratic coefficients were very significant, while the interaction between them was not (*p* = 0.937). The second order polynomial equation is the following:

$$\hat{Y} = 6,066 + 0,096 X_1 - 0,231 X_2 - 0,464 X_1^2 - 0,484 X_2^2 + 0,002 X_1 X_2$$

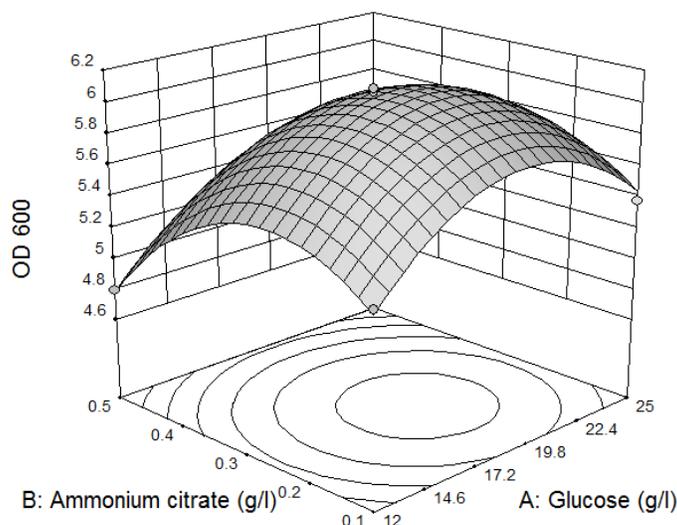


Fig. 2: Three-dimensional surface plot for the combination effect of glucose and ammonium citrate on *L. brevis* CHTD27 growth

where,

\hat{Y} = OD₆₀₀ value

X_1 = Glucose

X_2 = Ammonium citrate

Figure 2 shows the 3D Surface Plot of the OD₆₀₀ value against glucose and ammonium citrate. OD₆₀₀ value varies with the change of glucose concentration and found to be optimal at above 19.15 g/L. Meanwhile, ammonium citrate influenced highly the growth potential and gave the maximal growth at a concentration of 0.25 g/L.

Validation of the experimental model: *L. brevis* was cultivated on the optimized medium containing CIA and optimal concentrations of glucose and ammonium citrate obtained by the CCD (19.15g/L and 0.25 g/L respectively). The OD₆₀₀ value was found to be 6.00 (mean value) which is close to the predicted value of 6.098, confirms the adequacy of the model.

Comparison of the optimized CIA medium and MRS medium efficiency for *L. brevis* growth: The growth of *L. brevis* on the optimized medium was compared to that obtained on the MRS standard medium usually used for *Lactobacillus* growth. The results showed that the final OD₆₀₀ value reached in the optimized medium after 24 h of incubation was 6.00 (mean value) and surpassed that obtained in MRS medium which showed an OD₆₀₀ value of 4.53. Thus, the performance of the optimized medium was 32% higher than that of MRS medium; this may be explained by the richness of CIA in protein, the affinity of its peptidic fragments for LAB growth (as described earlier) and the addition of glucose as a carbon source. Moreover, CIA medium was formulated especially to support the growth of *L. brevis* by the selection of the required components and the removal of those having

negative effects on its growth. This was not the case for MRS medium; indeed, MRS was designed to support growth of the most fastidious LAB and consequently, some of its components may not be required for the growth of less nutrient-demanding species growth such as some lactobacilli (Horn *et al.*, 2005).

CONCLUSION

Chicken intestine is regarded as an attractive source of protein presenting the advantage to be so abundant and not exploited. That is why; their use in the formulation of a growth medium for LAB can constitute a good way for their valorisation. The optimal growth of the strain was reached on a medium composed of CIA supplemented with glucose and ammonium citrate. This growth surpassed that obtained on the commercial medium MRS, hence, CIA produced from an animal by-product using a low-cost method deserves to replace efficiently the MRS medium composed of complex and expensive sources for *L. brevis* growth.

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