



# Best Conditions for the Production of Natural Isopentyl Acetate (Banana Aroma) from Cheese Industry Waste: An Experimental Precursor Approach

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**Abstract:** In some fermentation systems, whey components (lactose, proteins and minerals) can produce isopentyl acetate (IA). An analysis of the best conditions for IA production with *Kluyveromyces marxianus* was developed in this work. The experiment design was two-factor and three-level design based on a response surface methodology (RSM) using Design-Expert<sup>®</sup> software. The analysis of anomeric protons by nuclear magnetic resonance (1H-NMR) showed 81.25% of  $\beta$  lactose content. This characteristic favored the production of IA. The maximum output (Mp) of IA, determined by gas chromatography, was 9.52 g/L (p < 0.05). The central composite design (CCD) was used to perform the factor analysis. Results showed that concentrations of 0.03 (g/L) ammonium sulphate and 0.3 (v/v) of isoamyl alcohol are the best conditions for a maximum rate of IA production. The production of IA can reduce the discharge of whey, allowing its reuse and revaluation.

**Keywords:** waste valorization; dairy industry; whey fermentation; anomeric protons; central composite design

# 1. Introduction

The effluent from cheese production in the dairy industry significantly impacts the environment because of its physicochemical characteristics [1]. It has been reported that the dairy industry generates a significant amount (400,000 million liters) of wastewater per year worldwide; whey is 50% of this amount [1,2]. As it has been reported, the biological treatment of whey through a conventional aerobic process is very costly, US \$0.50/kg COD (chemical oxygen demand) [3]. Whey could be a renewable source to exploit, considering its high production (180 million tons) globally, due to more than 80% volume of processed milk. Among the main components of whey are the high lactose content (30–60 g/L, w/v) and 20% (w/v) protein, which contains approximately 50% of the present nutrients in milk [1]. The lactose content present in whey exceeds the limits set by the national and international regulations, generating adverse effects on the environment [4]. For most of the twentieth century, the industry has sought the cheapest whey disposal method, which has generally involved discharging it into waterways, municipal wastewater treatment, or open fields [3].



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**Copyright:** © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). Conde et al. [5] presented an analysis on the content of  $\beta$ -lactose in sweet whey, which demonstrated advantages over the production of metabolites of industrial interest. This conformation could promote the formation of isopentyl acetate (IA), a short-chain fatty acid ester characterized by a strong banana smell [6]. Nowadays, the production of IA is mainly through the Fischer esterification method, which has among its disadvantages that the ester produced cannot be labeled as natural [7]. The toxicity of organic products is originated from the solvents used, the utilization of acids as catalysts, and the difficulty in extracting the compounds of interest [8]. On the other hand, the consumption of "natural" IA is increasing (75 tons per year) in industries such as pharmaceuticals and food mostly [9,10]. The "natural" aromas can be obtained only by: physical, enzymatic, or microbial processes, biocatalysis, novo synthesis (fermentation), and plants isolation [7]. Europe and the United States enable their safe use as additives [8,9].

The biochemical production of IA has been achieved mainly through the synthesis of enzymes (lipases and esterases), reaching concentrations of up to 4 g/L (v/v) [8,9]. It has been reported that enzymatic synthesis is highly attractive. However, it results in selective processes that must have temperature control [11]. The novo synthesis is the preferential route to increase the number of fruity esters from sugars and alcohols in yeasts [12]. IA has been produced from various synthetic substrates (cassava bagasse, molasses, peptone, yeast extract, glucose, etc.). To the best of our knowledge, there is no report on the improvement of IA production in whey with the addition of ammonium sulfate and isoamyl alcohol. Consequently, in this work, the best conditions to increase IA production were determined by whey fermentation using *Kluyveromyces marxianus* yeast isolated from *Agave durangensis*. Biotechnological production would be an alternative to obtaining IA, contributing to the revaluation of the dairy industry residues, which harm the environment.

# 2. Materials and Methods

### 2.1. Yeast Strain

The K.marxianus ITD00262 strain, from the private collection of the Durango Institute of Technology, isolated by Páez et al. was used [13]. The strain was preserved on plates using Standar Methods Agar (Bioxon <sup>TM</sup>-Guadalajara, Mexico) at 4 °C until use.

# 2.2. Whey Preparation

The samples were taken from the production of cheese. The dairy serum contained  $0.73 \pm 0.05$  (%, w/v) protein;  $7.58 \pm 0.04$  (%, w/v) easily-assimilable nitrogen (EAN);  $0.49 \pm 0.082$  (%, w/v) fat;  $5.21 \pm 0.15$  pH;  $51.24 \pm 1.51$  total suspended solids (TSS, mg/L) were determined via an infrared spectrophotometric technique, using the Lactoscan MCC equipment (Milkotronic Ltd., Nova Zagora, Bulgaria). Whey was previously prepared by a LTLT process (Low Temperature, Long Time) (63 °C/30 min). Subsequently, pH was adjusted to 4.8 using 1 N H<sub>2</sub>SO<sub>4</sub>, and the pH was determined according to the norm NMX-F-317-S-1998. This step was performed at kinetics beginning to activate the lactose permease Lac12p and Lac4  $\beta$ -cytosolytic galactosidase that hydrolyzes lactose into glucose and galactose [14]. The proximate analysis of whey was performed according to the Kjeldahl method of the AOAC 930.52. 25 mL of whey was settled in digestion tubes (250 mL) [14].

#### 2.3. Isopentyl Acetate Production

Whey, inoculated with  $1 \times 10^6$  cells/mL of K. marxianus, was fermented in 250 mL bottles. It uses an isothermal process (28 °C) of constant volume (150 mL). The flasks were incubated in agitation (180 rpm) at 28 °C for 96 h (Shaker Thermo<sup>®</sup> TM MaxQ<sup>TM</sup> Scientific, Santa Clara, CA, USA). Every 24 h, aliquots of 1 mL were taken from each flask, placed in sterile plastic (Eppendorf, Monterrey, Mexico) tubes, and stored at -20 °C until being analyzed by gas chromatography. The samples were analyzed in duplicate. The strain was pre-cultured in the medium, and was preserved in agar plates with standard

methods (SMA) (standard methods (Bioxon, Guadalajara, Mexico-Agar) according to Conde et al. [14]. It was incubated for 12 h while maintaining an agitation of 1 Relative Centrifugal Force unit (RCF) or g-force at 30 °C (Shaker Thermo<sup>®</sup> Scientific, Santa Clara, CA, USA).

#### 2.4. Monitoring Process

A yeast count was periodically performed from each flask by a viable stain with methylene blue (Innovating Science<sup>TM</sup>, Rochester St., Avon, NY, USA) in a Neubauer chamber (Marienfeld-Superior<sup>TM</sup>, Lauda-Königshofen, Germany). The lactose content throughout the fermentation was determined by the dinitrosalicylic acid (DNS) technique adapted from Horstch et al. [15]. Samples were prepared in triplicate, and centrifuged for 6 min before analysis. The equipment used was a centrifugal 9677 RCF (Eppendorf <sup>TM</sup>, Hamburg, Germany).

For the analysis of IA, the samples were centrifuged at 9677 RFC for 3 min in Eppendorf<sup>®</sup> tubes. Subsequently, the samples were microfiltered and analyzed by gas chromatography with flame ionization detector (FID) (GC; Perkin Elmer<sup>®</sup>, Santa Clara, CA, USA) equipped with a J&W Scientific<sup>®</sup> DB-WAX column, Santa Clara, CA, USA (60 m × 0.25 mm × 0.25 microns). The injector, detector, and oven worked at 230 °C, 250 °C, and 140 °C, respectively. The extract of the injected samples was 1  $\mu$ L. Reference standards were used for the analysis of the samples. The samples were analyzed in triplicate.

#### 2.5. 1H-NMR

For the analysis by nuclear magnetic resonance (NMR), the samples were previously centrifuged at 9677 RCF (Eppendorf<sup>TM</sup>, Hamburg, Germany) for 6 min. Subsequently, a LTLT process was applied (60 °C/30 min). 1H-NMR spectra were obtained with a Varian<sup>®</sup> 400 MHz spectrometer, Arizona, United States. Deuterium oxide (D<sub>2</sub>O) was used as a solvent. Five scans were performed to analyze proton NMR with a sequence zg30 (30° pulse before the evaluation of the spectrum) in an NMR Varian<sup>®</sup> equipment, 400 MHz [12].

## 2.6. Optical Rotation

This analysis was determined with a Perkin Elmer<sup>®</sup>, Inc 341 Polarimeter to determine the optimal rotation present in whey samples, complementing the NMR study. The equipment is provided with a sodium lamp adjusted to monochromatic radiation of  $\lambda = 589$  through a 1 dm cell. The samples were prepared with 40 mL of sweet whey placed in conical propylene tubes and centrifuged at 9677 RCF (Eppendorf<sup>TM</sup>, Hamburg, Germany) for 10 min. The remainder was placed in a 250 mL ball flask and evaporated on a Buchi Rotavapor Water Bath<sup>®</sup> Darmstadt, Germany, B-480 at 80 °C, using an average rotation. Subsequently, 60 mg from the extract were dissolved in 2 mL of dimethyl sulfoxide (DMSO-d6). The specific rotation was calculated as follows:

$$\alpha_{obs}]_D = \frac{100^\circ \propto}{1 \times C} \tag{1}$$

where:

 $\alpha$  is the optical rotation

*D* is the monochromatic radiation sodium ( $\lambda = 589$  nm)

1 is a decimeter long

*C* is the sample volume

#### 2.7. Mathematical Modeling

The mathematical modeling was determined for the maximum rates of production (rp) of IA from each treatment by correlating the experimental data of accumulated production at time t. Additionally, Gompertz differential equations for non-linear regression and the Levenberg-Marquardt algorithm according to Deseure et al. [16] were applied. The values of the coefficients of equation 2 were determined according to Conde et al. [5]. These

values were used to obtain the coefficients (A,  $u_m$  and  $\lambda$ ) corresponding to the solution for Equation (3) [16]:

$$y = a \cdot exp[-exp \ exp \ (k - xc)] \tag{2}$$

Solution of the Gompertz equation to  $P_{max}$ ,  $r_p$  and  $\lambda$ , where:

 $P_{max}$  is the maximum production of IA obtained (g/L)

 $r_p$  (k/e) is the maximum rate of formation of IA ( $h^{-1}$ )

 $(xc-a/r_p\lambda \cdot e)$  is the cell duplication time (h<sup>-1</sup>) and the Euler constant.

$$y = P_{max} \cdot exp\left\{-exp\left\{\frac{r_p \cdot e}{P_{max}}\right\}(\lambda - t) + 1\right\}$$
(3)

#### 2.8. Scanning Electron Microscopy (SEM)

The samples contained in Eppendorf<sup>®</sup> Safe-Lock Microcentrifuge tubes were prepared in a lyophilizer console (Labconco<sup>®</sup>, Kansas City, MO, USA). Freeze drying conditions were at -52 °C (capacitor) and vacuum pressure of 0.05 mbar. Samples were lyophilized for five hours and then prepared for SEM imaging, using a JEOL<sup>®</sup> JSM-6300 scanning electron microscope, with a current probe of 02/10 to 10/05 amperes (A) and a power of 2 to 30 kiloelectron volts (keV).

# 2.9. Statistical Analysis

The CCD of two factors and three levels was applied in the study to find by optimizing conditions for IA production (see Table 1), using Design<sup>®</sup> Expert software (7.1.4.0) for the construction of a response surface evaluating the independent variables, and concentration of ammonium sulphate and isoamyl alcohol. The source of nitrogen available in whey for *K. marxianus* is 25%; due to this, the utilization of ammonium sulfate used for its bioconversion in the Novo synthesis was considered [14].

Table 1. Levels of the factors used in the optimization of the maximum production rate of isopentyl acetate.

Factor	Independent Variables	Level				
		-1.414 (-α)	-1	0	1	1.414 (+ $\alpha$ )
X <sub>1</sub>	Isoamyl alcohol $(v/v)$	0.26	0.3	0.4	0.5	0.54
X <sub>2</sub>	Ammonium sulphate (g/L)	0.026	0.03	0.04	0.05	0.054

As a dependent variable for this case, the maximum IA production rate was considered (see Table 2). The distance for each axial point was 1.68 ( $\alpha = 2^{k/4}$ ), considering k as the number of significances of the independent variables, while the number of experiments of such variables was calculated as 20 (2k + 2K + 4) [17]. The analysis of the variables was carried out according to the following equation:

$$X_1 = \left(U_i - U_I^0\right) \Delta U_i \tag{4}$$

where:

 $X_1$  is the independent variable codification,

 $U_i$  represents the real value of the independent variable

 $U_I^0$  is the center point independent variable

Dun	Isoamyl Alcohol (X <sub>1</sub> )		Ammonium Sulphate (X <sub>2</sub> )		P <sub>max</sub> <sup>a</sup>	(g/L)	rp <sup>b</sup>	(g/L h)
Kun	Actual (v/v)	Code	Actual (g/L)	Code	Observed	Predicted		Actual (v/v)
1	0.4	0	0.04	0	7.73	7.64	0.59	0.60
2	0.4	0	0.054	1414	4.68	5.96	0.42	0.46
3	0.3	$^{-1}$	0.05	1	8.02	7.14	0.30	0.31
4	0.3	$^{-1}$	0.03	-1	9.95	9.52	0.26	0.34
5	0.4	0	0.04	0	8.12	7.64	0.56	0.60
6	0.5	1	0.03	-1	8.57	8.14	0.57	0.58
7	0.4	0	0.026	1414	8.37	9.32	0.59	0.53
8	0.4	0	0.04	0	7.65	7.64	0.69	0.60
9	0.4	0	0.04	0	7.96	7.64	0.57	0.60
10	0.5	1	0.05	1	6.22	5.76	0.58	0.51
11	0.26	-1.414	0.04	0	7.47	8.62	0.28	0.22
12	0.4	0	0.04	0	8.76	7.64	0.60	0.60
13	0.54	1.414	0.04	0	5.79	6.66	0.48	0.53

Table 2. Speed of specific production of isopentyl acetate in different proportions of isoamyl alcohol and ammonium sulphate.

 $Pmax^a$ : is the maximum IA production reached (g/L), rpb: is the maximum rate of isopentyl acetate formation.

In the optimization process, the response variable may relate to independent variables chosen through the following quadratic model:

$$Y_i = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$
(5)

where:

 $Y_i$  values are the response predictions

 $x_i$  are the parameters

 $\beta_0$ ,  $\beta_i$ ,  $\beta_{ii}$  are the constant values. While  $\beta_{ii}$  are the interactions of the coefficients.

The results were analyzed through a one-way variance analysis (ANOVA) by Fisher's Least Squares Deviation. The data were analyzed with the STATISTICA<sup>®</sup> software 8.0 considering a confidence level of 95%. The least significant difference formula is shown below:

$$LSD_{A,B} = t_{\frac{0.05}{2}, DFW_{\sqrt{msw(\frac{1}{n_{a}} + \frac{1}{n_{b}})}}}$$
(6)

where:

*t* is the critical value obtained from tables *msw* is the average of the ANOVA value *n* is the number of tests

#### 3. Results

The content of initial lactose for whey resulting from the casein coagulation by the action of renin at pH 6.5 was 54 g/L (w/v). Some authors have reported similar lactose concentrations for this type of whey ranging from 46 to 56 g/L (w/v) [18]. Furthermore, the anomeric conformation for substances with lactose was almost not reported [18]. In this paper, the whey extraction with DMSO resulted in a white solid (14 mg, 0.17%) that showed an optical rotation of +28, using monochromatic radiation of  $\Lambda$ -589 nm at 20 °C. These results indicate the presence of a dextrorotatory molecule. The solid was physically and spectroscopically characterized; additionally, it was compared with data for (+)- $\beta$ -lactose and (+)- $\alpha$ -Lactose mixture [19].

It was determined using NMR spectrum that the content of  $\beta$ -lactose was 81.25%  $\pm$  0.81 and 0.36%  $\pm$  17.64 for  $\alpha$ -lactose. These results are associated with the pH of sweet whey that favors the nucleation process by increasing the conversion rate of  $\alpha$  and  $\beta$  anomers, increasing solubility [7,19]. Conversion between these forms of lactose is highly dependent on temperature, the presence of salts, and lactose concentration in whey [19]. Scanning electron microscopy (SEM) showed the microstructure of whey as a matrix of rough ap-



pearance of the porous and open protein network. In this matrix, numerous fat globules of varying size and hemispherical shape were uniformly distributed (Figure 1).

**Figure 1.** Micrograph of the whey microstructure: (**a**) without biofilm *K. marxianus* and (**b**) scanning of the optimal conditions for IA production in whey, time of 48 h *K. marxianus* biofilm formation.

The presence of the proteins in whey through the water-binding contributes to the nucleation process, creating areas of saturation. This process has already been described through some kinetic models [20]. The phase transitions and their effect on matrix properties analysis allow establishing a factorial design. Then, the conditions improve the *K. marxianus* intracellular enzyme performance, allowing the use of lactose as a carbon source [21]. This favored the IA production increase.

Gas chromatography (GC) showed a maximum production ( $P_{max}$ ) 9.56 to IA g/L (treatment 4) at 48 h. These results contrast to those by Texeira et al. [22], who obtained an IA concentration of 0.17 g/L. On the other hand, Calleros et al. [23] obtained an IA concentration of 0.23 g/L in serum of whey (tofu). The results could be caused by the  $\beta$ -lactose content and ammonium sulphate with the supplement as EAN source promoting the alcohol acetyltransferase (AATase) metabolic pathway [24]. This route, which generates the production of esters from fatty acids present in whey during fermentation, allows the formation of IA by transfer of functional groups [25]. In addition, the increase in IA production was favored by adding isoamyl alcohol through the metabolic pathway to both the synthesis de novo and the Ehrlich Pathway [7,9]. Conde et al. [5] showed the production of ethanol in sweet whey of 15.59 (g/L) in the production of 2-phenylethanol (2-PEA). In this work, a concentration of 6.24 g/L was obtained (24 h). The high production of ethanol has been reported to contribute to the formation of acetaldehyde converted to isoamyl aldehyde and finally reduced to IA through the route that involves the formation of higher alcohols [21].

The optimum levels of the key factors in the production rate of isopentyl acetate were determined by design (RSM) response surface methodology. Table 3 shows the specific results of the experimental design of the variable design matrix.

Source	Sum of Squares	$D_{f}$	Mean Square	F-Value	<i>p</i> -Value
MODEL	0.2044	5	0.0409	8.6	0.0067
X <sub>1</sub>	0.0977	1	0.0977	20.55	0.0027
X <sub>2</sub>	0.0049	1	0.0049	1.03	0.3442
$X_1X_2$	0.0002	1	0.0002	0.0398	0.8476
X <sub>12</sub>	0.091	1	0.091	19.13	0.0033
X <sub>22</sub>	0.02	1	0.02	4.21	0.0793
Residual	0.0333	7	0.0048		
Adjustment	0.0233	3	0.0078	3.09	0.1519
Pure Error	0.01	4	0.0025		
Cor Total	0.2377	12			

Table 3. ANOVA results for the quadratic polynomial model response surface.

R<sup>2</sup>: 90.0%, Coefficient of variation (CV): 13.0%.

A second-order polynomial regression equation shows the dependence of the maximum rate of formation of ammonium sulphate and IA with the isoamyl alcohol. The multiple regression analysis of the experimental data allowed us to determine the parameters used in the following polynomial equation:

$$Y = 0.603 + 0.110x_1 - 0.024x_2 - 0.006x_1^2 - 0.114x_2^2 - 0.053x_1x_2$$
(7)

where:

*Y* is the prediction of the response variable

 $x_1$  are the values of the independent variables

 $X_1$  (isoamyl alcohol) and  $x_2$  is the encoded value of the variable  $X_2$  (ammonium sulphate)

This equation was optimized by using the method of interaction (SigmaPlot 12.5 software). The model coefficients were estimated by linear regression, where the value of (p) was required for assessing the significance of each coefficient. In this work, the individual effects of X1 and  $X_1^2$  showed significance (p < 0.05) for the production of IA, with a coefficient estimated at 0.11  $X_1$  and -0.006 to  $X_1^2$  Equation (7). The higher the value of (t) and the lower the value of (p), the value of the corresponding coefficient has greater significance [26]. The significance of the coefficients was determined through the Student t-distribution, while the p values are shown in Table 3.

The validation of the model was carried out through the analysis of the residuals with a confidence interval of 95%; the significant differences of the statistical model were determined by means of an analysis of variance (ANOVA) F-test. The regression adjustment is still significant at p < 0.005, which is adequate to represent the relationship between IA production and the independent variables. The coefficients estimation by a linear regression model, based on the effect of the variables, resulted in the following equation:

$$Y = 7.64 - 0.694x_1 - 1.19x_2 \tag{8}$$

where:

*Y* is the prediction of the response

 $x_1$  is the encoded value of the variable  $X_1$  (isoamyl alcohol), and  $x_2$  is the encoded value of the variable  $X_2$  (ammonium sulphate)

The independent variables showed optimal values for achieving the maximum rate production of IA of 0.63 g/L h are 0.45 (v/v) for  $X_1$  and 0.037 (g/L) for  $X_2$  the quadratic model for rp; Pmax was obtained at maximum concentration with the linear model of 9.52 g/L in  $X_1 = 0.3$  (v/v) and  $X_2 = 0.03$  (g/L) (Figure 2).



**Figure 2.** The response surface plots showing the effects of independent variables on the  $r_p$ .

According to the two-dimensional contour plot, the process can be more sensitive to changes in the concentration of isoamyl alcohol than to changes in the concentration of ammonium sulphate, with a coefficient estimated at -1.19 to X<sub>2</sub> Equation (8). For these conditions, the scanning electron microscope (SEM) showed a time of 48 h, where it has the highest rate of IA production and the formation of biofilm *K.marxianus*, with a  $6.98 \times 10^6$  count CFU/mL. This could be due to protein aggregates of tiny particles of heat-coagulated whey protein, with diameters of 1 to 2  $\mu_m$  [27]. The *K. marxianus* microstructure shows several similarities to that reported by Cabaroglu et al. [28].

## 4. Conclusions

In this study, the most favorable conditions reported to date were found for the biotechnological production of isoamyl acetate, a product of high commercial value, from whey. Ammonium sulphate and isoamyl alcohol concentrations were analyzed as independent variables in IA production. The data were analyzed using the response surface technique (RSM). The concentrations of 0.3 (v/v) of isoamyl alcohol and 0.03 (g/L) of ammonium sulfate generated the maximum concentration of IA, which corresponded to 9.52 g/L. So far, this is the highest reported IA concentration value at the exit of a fermentation. The bromatological and SEM analysis of whey identified three critical issues: whey physicochemical properties, the enzymatic coagulation type, and the protein concentration. Sweet whey is a good source of carbon in fermentation processes. Compared with other types of whey (acid/curd), sweet whey has high  $\beta$ -lactose content, increasing its solubility and allowing its bioconversion to interest metabolites at an industrial level. The presence of this dextrogyrous molecule that participates in the nucleation process was validated with polarimetry analysis. Author Contributions: Contribution: investigation, C.A.G.-A., J.C.-R., A.L.-M., C.C.-M., A.J.-G., S.A.M.-M. and L.C.-B.; methodology, C.A.G.-A., C.F.P.-M., M.P.F.-L. and L.C.-B.; visualization, C.A.G.-A. and J.C.-R.; writing—original draft, A.L.-M., C.C.-M. and L.C.-B.; writing—review and editing, A.L.-M., C.C.-M. and L.C.-B. All authors have read and agreed to the published version of the manuscript.

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