

# Evaluation of alternative microbial transglutaminase production from sorghum grain and distilled dried grains with solubles using computational simulation

Evaluación de la producción alternativa de transglutaminasa microbiana en medios a base de granos de sorgo y granos secos de destilería utilizando un simulador computacional

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## ARTICLE INFO

Received June 17, 2015

Accepted April 07, 2016

## KEYWORDS

Transglutaminase, simulation, sorghum, DDGS

Transglutaminase, simulación, sorgo, DDGDS

**ABSTRACT:** Microbial transglutaminase (MTGase) is widely used as an additive in the food industry for improving the mechanical properties and texture of foods. The enzyme production process is expensive due to the cost of the components of the fermentation medium used, therefore, it was evaluated the possibility of using inexpensive raw materials for *Streptomyces mobaraensis* growth. An economic study of microbial transglutaminase (MTGase) production with a variety of raw materials would be a long process. The software *SuperPro Designer*® v7.5 was used, based on data obtained from pilot plant scale fermentations, to estimate the services, capital, and operating costs, and revenue of the product. The model showed an estimation of MTGase production costs using glucose obtained by enzymatic hydrolysis of sorghum grains supplemented with distilled dried grains with solubles (DDGS). The MTGase production plant using enzymatic hydrolysates yielded an annual gross income of  $12.326 \times 10^6$  US\$ and a return on investment time of 4.01 years.

**RESUMEN:** La enzima Transglutaminasa microbiana (MTGasa) es ampliamente usada en la industria alimentaria como aditivo para mejorar las propiedades mecánicas y de textura de los alimentos. El proceso de producción de la enzima es caro debido al valor de los componentes del medio de fermentación utilizados, por ello se evaluó la posibilidad de utilizar materias primas baratas, como fuente de nutrientes para el crecimiento de *Streptomyces mobaraensis*. El estudio económico de la producción de MTGasa con una variedad de materias primas sería un proceso largo. El software de simulación de procesos industriales *SuperPro Designer*® v7.5 fue utilizado, basándose en datos obtenidos de fermentaciones a escala de banco, para estimar el consumo de servicios, costos de capital, costos de operación e ingreso por el producto. El modelo mostró el estimado de los costos de producción de MTGasa utilizando glucosa obtenida de la hidrólisis enzimática de granos de sorgo y suplementada con granos secos destilados con solubles (DDGS por sus siglas en inglés, *Dried Distillers Grains with Solubles*). En la planta productora de MTGasa, utilizando hidrolizados enzimáticos, se obtuvo una ganancia bruta anual de  $12.326 \times 10^6$  US\$ y un tiempo de recuperación de la inversión de 4.01 años.

## 1. Introduction

Transglutaminases (TGases, EC 2.3.2.13) are a family of proteins that are present in most tissues and extracellular fluids of vertebrates and are widely distributed in nature [1]. These enzymes were identified in the liver of guinea pigs [2]. Unlike most industrial enzymes—amylases, lipases and

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ISSN 0120-6230  
 e-ISSN 2422-2844

proteases—they catalyze hydrolysis reactions and induce the polymerization of proteins. Consequently, they are able to modify the functional properties and texture of proteins creating products with better consistency and better quality; some enzymes are being capable of incorporating amines within proteins [3, 4]. TGase is widely used in the food industry to obtain products of greater quality with a better nutritional content; the enzyme is used in seafood, surimi products, meat products, noodles/pasta, dairy products, baked goods, etc. [5-7]. Production of this enzyme is currently carried out by bacteria, with *Streptomyces* species being the most commonly used [8]. Obtaining TGase using microorganisms has the advantage of providing better yield compared to that obtained from the liver or serum of guinea pig, which were the primary sources for obtaining the enzyme [9]. In addition, microbial transglutaminase (MTGase) is a calcium-independent enzyme which also makes it more useful in food processing and production; the enzyme also has a wide pH range (4 to 9) and temperature (0 to 55°C), which increases its advantages [10-13]. However, despite all the advantages and uses of MTGase, its production is an expensive process, mainly because the components of the fermentation medium represent approximately 30% of the total cost of the process [14]. Therefore, some researchers have focused their attention on finding cheaper nutrient sources for production of the enzyme, especially the carbon source, since it is essential for bacteria growth.

The use of sugarcane molasses and xylose obtained from acid hydrolysis of sorghum straw meal has been studied [14]; both are raw materials that are considered waste and inexpensive carbon sources; however, they have not yielded the expected results in enzyme activity. Therefore, it is important to continue investigating new available raw materials that could produce suitable carbon sources for the growth of bacteria and MTGase production. In Mexico, sorghum is an important grain in agriculture, but it is used almost exclusively for feeding cattle. In contrast, corn is used mainly as food for humans. Tamaulipas state ranks first in the production of sorghum and maize; however, these crops are sold at a low price [15]. Therefore, the use of these grains to obtain fermentable sugars for growth of microorganisms and production of metabolites would reduce the cost of the process and also, would add greater value to these grains. It is possible to obtain by enzymatic hydrolysis a high concentration of glucose from grain flour and replace glycerol as a carbon source for the growth of *Streptomyces mobaraensis* with the subsequent production of MTGase.

The feasibility of producing MTGase from sorghum grains supplemented with dried distillers grains (*Dried Distillers Grains with Solubles*, DDGS) should be evaluated and compared to the enzyme produced using the common medium [8]. *SuperPro Designer®* software v. 7.5, which is used worldwide to design and simulate the production of chemicals and pharmaceuticals, was used to perform the simulation of a plant and the evaluation of MTGase production with the culture medium. The objective of this study was to evaluate the economic feasibility of MTGase production using grain sorghum and DDGS.

## 2. Material and Methods

### 2.1. Preliminary experiments to obtain enzyme with different culture media

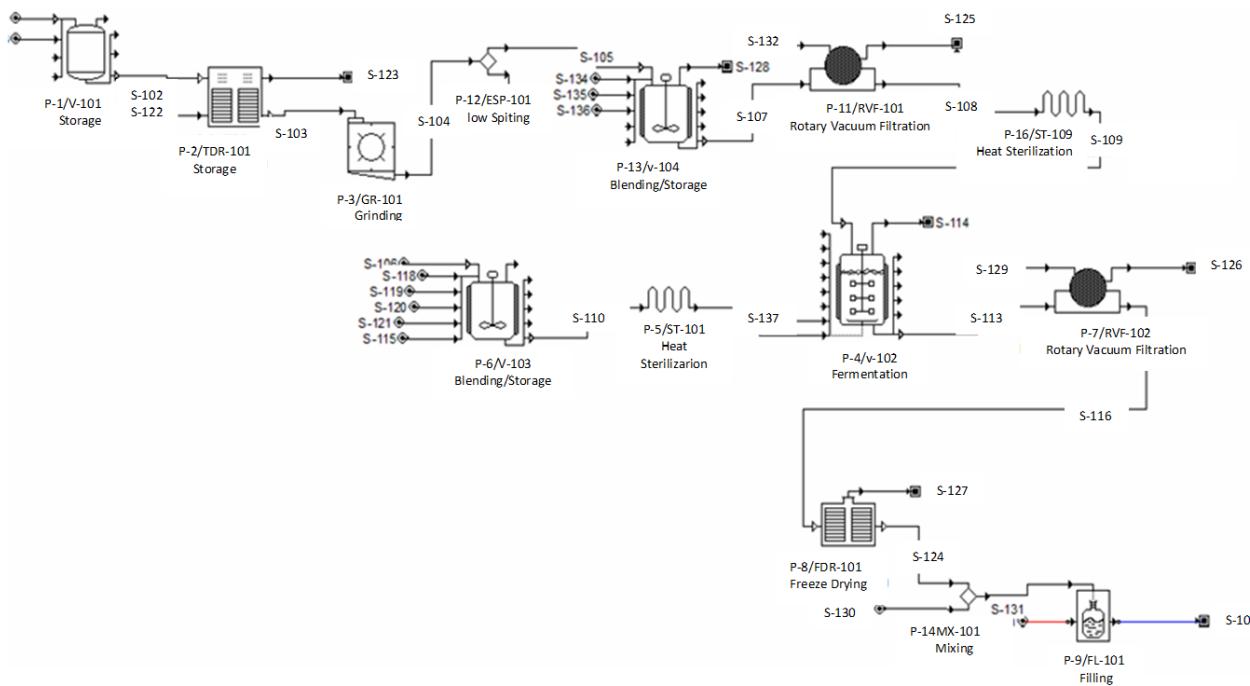
Fermentations were conducted with different culture media to determine the best composition of components and increased production of MTGase. A Box-Behnken experimental model was performed for culture media with three factors and three levels of 72 and 96 h of fermentation. Three different concentrations of casein, yeast extract, and DDGS were tested. Concentrations of glucose 30 g/L,  $MgSO_4$  0.5 g/L,  $KH_2PO_4$  g/L,  $Na_2HPO_4$  g/L, and peptone 10.5 g/L were kept constant in all media.

To measure MTGase activity, the Grossowicz method was used [16]. The procedure for measuring MTGase enzyme activity was as follows: 5 mL of culture medium was taken at different inoculation times (72, 96 and 120 h), and these were centrifuged at 7000 rpm/10 min. An aliquot of 0.2 mL of supernatant was taken from each tube; one tube was placed in a water bath at 75°C to inactivate the enzyme and this was the blank. Subsequently, we added 0.5 mL of the solution of the specific substrate N-CBZ-GLN-GLY (Sigma-C6154, Sigma Aldrich Co., St. Louis, MO) to the tubes with the sample and blank and incubated these at 37°C for 10 min. At the end of this time, the reaction was quenched with 0.2 mL of 5%  $FeCl_3$  solution in 0.1 N HCl and the absorbance was measured at 525 nm. Calculations were made to obtain the MTGase units using a calibration curve of monohydroxamate. For this, commercial MTGase (Ajinomoto Co. Inc.) was used as a control. In this method, a unit of enzyme is defined as the formation of 1 mol of hydroxamate per minute at 37°C.

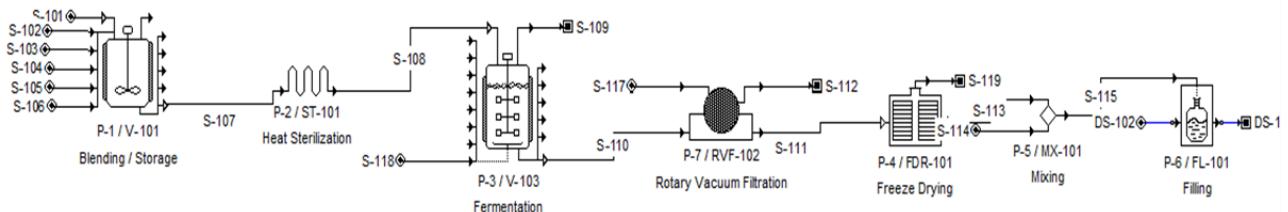
The experimental design followed an incomplete and second-order factor structure. The yeast extract, DDGS, and casein were considered independent variables and their effects on the dependent variables (TGase activity at 72 and 96 h) were calculated. The interaction between medium composition variables and dependent variables can be set through a linear equation including linear, interaction, and second-order terms. In the experimental design, encoded factors were named X1 (casein), X2 (yeast extract), and X3 (DDGS).

### 2.2. Process simulation

The design and programming of the production plant of microbial transglutaminase from enzymatic sorghum grains hydrolysates was performed with *SuperPro Designer v. 7.5* (Intelligen Inc., Scotch Plains, NJ). The general process for obtaining enzyme using enzymatic sorghum flour hydrolysates is as follows: the raw material is treated by washing with running water, then it is dried and the sorghum grains are ground to obtain flour. The bran is separated from the flour and afterwards enzymatic hydrolysis is performed with  $\alpha$ -amylase and glucoamylase. After hydrolysis, the glucose juice is separated from the solid waste; glucose is sterilized by autoclaving (121°C for 15 minutes). In another



**Figure 1 Flowsheet for MTGase production with sorghum hydrolysates**



**Figure 2 Flowsheet for MTGase production with common media**

tank, the remaining components of the medium (peptone, yeast extract, casein,  $MgSO_4$ ,  $K_2HPO_4$ ,  $NaH_2PO_4$ ) are mixed. After mixing, these compounds were also sterilized to prevent the Maillard reaction whose products may inhibit bacterial growth. Once sterilized, both parts of the medium are mixed in a fermenter and fermented for 96 hours; the crude extract with the enzyme is separated from the solid residues by centrifugation. After MTGase is separated, it is lyophilized and subsequently packaged (Figure 1 and Table 1). In addition to the simulation of this process, an enzyme production plant was designed with the common medium (Figure 2 and Table 1), which has glycerol as a carbon source; the composition of the medium was glycerol 32 g/L,  $MgSO_4$  0.5 g/L,  $KH_2PO_4$  g/L,  $Na_2HPO_4$  g/L, peptone 10.5 g/L, yeast extract 2.5 g/L and casein 38.4 g/L.

## 2.3. Economic Analysis

*SuperPro Designer* ® Version 7.5 was used for the economic analysis of the process design (Figure 2). The cost of equipment was obtained from the *SuperPro-Designer*

database or at the website <http://www.kitmondo.com>. The total equipment purchase cost was  $4.413 \times 10^6$  US\$ and  $3.901 \times 10^6$  US\$ for plant with hydrolysates and common media, respectively. This was the basis for calculating the cost of other components of the fixed capital investment such as installation, instrumentation, etc. These costs are shown in Table 2.

## 3. Results and discussion

### 3.1. Preliminary laboratory experiments

The culture media results were analyzed with the statistical program *Design expert v7* (Stat-Ease, Inc., Minneapolis, MN). Analysis of variance showed that the casein-yeast extract interaction had a significant effect ( $p \leq 0.0275$ ) in the production of the enzyme, and casein was the nutrient with the greatest effect ( $p \leq 0.0003$ ) since media without casein had an MTGase activity close to zero. The program also made a prediction based on the experimental values;

**Table 1 Key inputs and outputs design MTGase production plant with both media based on enzymatic hydrolysates**

Medium with hydrolysates	Common medium	Raw material/product
S-102		Sorghum grain (unclean)
S-103		Sorghum (clean)
S-114		Sorghum (clean and dry)
S-105		Sorghum flour
S-112		Sorghum bran (subproduct)
S-134		Amylase
S-136		Glucoamylase
S-135		HCl
S-107		Sorghum hydrolysate
S-108		Glucose syrup
S-125		Subproduct (2)
S-109		Syrup (esterilized)
S-115		DDGS
S-106	S-101	Casein
S-118	S-102	MgSO <sub>4</sub> and yeast extract
S-119	S-103	KH <sub>2</sub> PO <sub>4</sub>
S-120	S-104	Na <sub>2</sub> HPO <sub>4</sub>
S-121	S-105	Peptone
	S-106	Glycerol
S-110	S-107	Mix (nutrients)
S-137	S-118	S, ladakanum
S-113	S-111	Crude extract (with MTGase)
S116		MTGase
S-124	S-113	MTGase (lyophilized)
S-130	S-115	Maltodextrin
S-131	S-115	Packing
DS-102	DS-102	Mix: MTGase-Maltodextrine

because DDGS does not influence the model ( $p < 0.4275$ ), the lowest concentration (2 g/L) can be used. The model predicts that the optimal concentrations are DDGS 2 g/L, yeast extract 2 g/L, and casein 15 g/L. With these conditions, a casein concentration of 0.67 U/mL was obtained.

The experimental data showed an activity of 0.62 U/mL (Table 2), which is consistent with the prediction [14]; therefore, these nutrient concentrations were chosen for the plant design in the industrial simulator. It is important to point out that it is not a replica of the MTGase production plant but a design based on the MTGase production process from sorghum grains. For comparison and validation of the feasibility of production of the enzyme with enzymatic hydrolysate media of sorghum, an MTGase production plant with a common culture media, which has glycerol as a carbon source, was designed in the same simulator.

The laboratory experiments that formed the basis for the design of enzyme production plants yielded an enzyme activity of 0.62 U/mL for the enzyme produced with media based on sorghum hydrolysates (Table 3), while with

glycerol a maximal activity of 0.44 U/mL was obtained. However, the commercial enzyme from *Ajinomoto Co., Inc.* (Tokyo, Japan) has an average activity of 1 U/mL, thus this activity was considered for the enzyme produced in medium with glycerol to compare production with an established company. *Ajinomoto Co., Inc.* achieves this activity using a genetically modified *E. coli* and not a wild strain of *S. mobaraensis*.

### 3.2. Process Design and economical evaluation

Plant design was carried out following the above production process, considering from the washing of raw material to the packing of the lyophilized enzyme. The enzyme marketed by *Ajinomoto Co. Inc.* contains 99% maltodextrin and 1% MTGase with an approximate activity of 100 U/g and the cost of this product is approximately 70 US\$/kg. The final product of the MTGase production plant contains about 62 U/g at a cost of 35 US\$/kg. For comparative purposes and to truly establish the profitability of the MTGase production plant

**Table 2 Fixed capital estimate summary for the MTGase production (price in US\$)**

Total Plant Direct Cost (TPDC)	Plant with hydrolysates	Plant with common medium
Equipment purchase cost	4,413,000	3,901,000
Installation	1,310,000	1,096,000
Process piping	1,544,000	1,365,000
Instrumentation	1,765,000	1,561,000
Insulation	1,320,000	117,000
Electrical	441,000	390,000
Buildings	1,986,000	1,756,000
Yard improvement	662,000	585,000
Auxiliary facilities	1,765,000	1,561,000
<b>Total TPDC</b>	<b>14,018,000</b>	12,331,000
<b>Total Plant Indirect Cost (TPIC)</b>		
Engineering	3,504,000	3,083,000
Construction	4,906,000	4,316,000
TPIC	8,410,000	7,399,000
<b>Total Plant Cost</b>	<b>22,428,000</b>	<b>19,730,000</b>
Contractor's fee [A]	<b>1,121,000</b>	986,000
Contingency [B]	2,243,000	1,973,000
<b>CFC [A+ B]</b>	<b>3,364,000</b>	<b>2,959,000</b>

**Table 3 Experimental design with three factors and three levels**

Exp.	DDGS (g/L)	Yeast extract (g/L)	Casein (g/L)	X1	X2	X3	MTG activity (U/mL) 96 h
1	6.0	2.0	30.0	0	-1	1	0.62
2	2.0	6.0	30.0	-1	0	1	0.54
3	10.0	10.0	15.0	1	1	0	0.57
4	10.0	6.0	30.0	1	0	1	0.80
5	10.0	6.0	0.0	1	0	-1	0.02
6	2.0	10.0	15.0	-1	1	0	0.60
7	6.0	6.0	15.0	0	0	0	0.78
8	6.0	2.0	15.0	0	-1	0	0.52
9	10.0	2.0	15.0	1	-1	0	0.50
10	2.0	6.0	0.0	-1	0	-1	0.19
11	6.0	10.0	0.0	0	1	-1	0.10
12	6.0	2.0	0.0	0	-1	-1	0.09
13	2.0	2.0	15.0	-1	-1	0	0.62
14	6.0	6.0	15.0	0	0	0	0.79
15	6.0	6.0	15.0	0	0	0	0.69

using glucose as a carbon source obtained from sorghum grains, it was necessary to simulate an enzyme producing plant using the common medium, which has glycerol as the carbon source and a higher concentration of nitrogen sources than the medium supplemented with hydrolysates. Equipment for obtaining sorghum flour was not necessary from this plant. Table 2 to 4 show name, code, inputs and outputs for plants.

The total capital investment was  $27.103 \times 10^6$  US\$ with an equivalent production of 320,158 units/year. Meanwhile, the unit product cost was 16.06 US\$/entity with a total revenue of  $12.326 \times 10^6$  US\$ in addition to obtaining 4,347 US\$ /year from sales of subproducts (ground sorghum residue, solid residue from hydrolysis, and solid residue from fermentation), for a total annual income of  $12.330 \times 10^6$  US\$. The byproducts from enzyme production contain a

high protein and fiber content, which may be used as cattle feed supplement.

The sum of capital investment is analyzed formerly (Table 2) showing the total plant direct costs (TPDC) and indirect costs (TPIC). The TPC is estimated at  $22.428 \times 10^6$  US\$; adding contractor fees and contingency costs results in a direct cost of fixed capital of  $25.792 \times 10^6$  US\$.

The cost of sorghum was consulted at the Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food (SAGARPA) [17]. The cost of the other reagents from *Sigma Aldrich* was quoted in dollars. The estimated annual cost of the raw materials was 134,000 US\$, and in turn the annual operating cost was  $5.140 \times 10^6$  US\$ (Table 5). This total includes the cost of raw materials, cost of operators, lab fees, waste management, utilities, and the pending issues fund; in this, consumables, transportation, sales strategies expenses, maintenance, and others are considered (Table 4). This plant shows an annual income of  $12.36 \times 10^6$  US\$; of this  $12.3 \times 10^6$  US\$ correspond to MTGase sale and 4,000 US\$ to subproduct sales (0.20 US\$/kg). There is a profit margin of 58.31% and a percentage of return on investment of 24.96%. According to all these data, the return on investment time is 4.01 years.

The same process was conducted but with commonly used medium ingredients [8]. Although this reduces the number of equipment because enzymatic hydrolysis is not required, the total investment does not significantly decrease (Table 4). We estimated savings of 12% in comparison with the equipment estimated in the prior plant. Furthermore, the concentrations of some components of the medium, such as casein, are duplicated, which increases production cost. By comparing the costs of raw materials in both production plants, we have an increase of 244% due to the high cost of carbon and nitrogen sources in the common medium, compared with the medium based on sorghum hydrolysates with DDGS. In the plant with sorghum hydrolysates, the annual cost of raw material is estimated at 134,000 US\$, while the other plant's estimated cost is 327,000 US\$.

The plant design with common medium generated in the program is displayed in Figure 2. The total equipment purchase cost was  $3.901 \times 10^6$  US\$. As in the previous design, the purchase cost of the equipment was the basis for calculating the cost of other components of the fixed capital investment, such as installation, instrumentation, etc. These costs are shown in Table 2.

The prices of equipment and raw materials were consulted in the same sources as in the previous plant. The same number of operators for the process and the same number of hours and wages were used (Table 5). This plant shows an annual income of  $10.836 \times 10^6$  US\$ of which  $10.774 \times 10^6$  US\$ correspond to MTGase marketing and the remainder to the sale of fermentation by products (0.20 US\$/kg). A profit margin of 48.91% and a return on investment of 22.29% were defined. According to all the data, it was determined that the return on investment time is 4.49 years as shown in Table 6.

In the MTGase production plant based on sorghum hydrolysates, 4,916 kg of enzyme are obtained per year which yields 3,680 units/batch, while the enzyme produced with the common medium produces 4,550 units per batch with a production of 8,505 kg of MTGase per year. Although less equipment is used in the plant that produces the enzyme with glycerol as a carbon source, the cost does not decrease significantly due to the amount of some raw materials such as casein. In the medium with hydrolysates a casein concentration of 19 g/L is used, while in the medium with glycerol 38 g/L is used.

The return on investment in both plants is almost similar, as shown in Table 6, with a difference of 0.5 years less in the hydrolysates based production plant; moreover, the cost of MTGase produced by hydrolysates is less than the enzyme produced with the common medium, therefore it would have a higher demand. It is important to point out that it was assumed that an activity of 1 U/mL would be obtained using the common medium, although fermentations using this medium resulted in a maximum activity of 0.4 U/mL. The value of 1.0 U/mL was used because this is how enzyme activity is currently marketed by *Ajinomoto Co. Inc.*, although the strain used for the marketed enzyme is genetically modified to increase MTGase activity. Even though genetically modified strains increase production and have been shown to be safe in the production of food additives, it is important to have a thorough purification process to avoid the presence of toxic substances that may cause harm to health; therefore, the use of totally harmless wild strains such as *S. moharaensis* is preferable for obtaining food additives. The plant that used the hydrolyzed enzyme as a glucose source, reported a higher percentage of profits than the plant in which glycerol was used (58.3% and 48.9%, respectively). For the enzyme produced with enzymatic hydrolysates a cost of 35 US\$/kg was obtained; while the enzyme produced with the common medium was 70 US\$/kg,

**Table 4 Operating cost summary MTGase production plants (Price in US\$)**

Cost item	Plant with hydrolysates	Plant with common medium
Raw materials	134,000	327,000
Facility-dependent	4,871,000	4,285,000
Labor-dependent	114,000	114,000
Laboratory/QC/QA	17,000	25,000
Utilities	5,000	2,000

**Table 5 Important input and output economic parameters used in the SuperPro Designer**

Name/Description	Total	Unit cost US\$
Reception tank/storage	1	47,000
Tray dryer. Drying area 6.60 m <sup>2</sup>	1	65,000
Grinder	1	69,000
Heat sterilization (control tubes). Performance 564.17 L/h	2	359,000
Agitation tank. Capacity 290 L	2	294,000
Lyophilizer. Sublimation rate 686.18 kg	1	\$2,000,000
Rotary vacuum filter. Filter area 0.53 m <sup>2</sup>	1	\$33,000
Rotary vacuum filter. Filter area 2.78 m <sup>2</sup>	1	\$50,000
Fermenter. Capacity 896.34 L	1	\$594,000
Labor (base salary)	4	6.5 h-1
MTGase (Media with hydrolysates)		35 Kg-1
MTGase (Common media)		35 Kg-1
Subproducts		0.20 Kg

**Table 6 Economic comparison of two plants**

Concept	Plant with hydrolysates	Plant with common medium
Gross sale (US\$)	12,326,000	10,836,000
Gain margin (%)	58.31	48.39
Return on Investment (%)	24.96	22.29
Investments return (years)	4.01	4.49

which is its current market price. These results confirmed the feasibility of MTGase production using glucose as a carbon source obtained from enzymatic hydrolysis. A culture medium with 600g/L skim milk, 40g/L potato and 5g/L glycerol was evaluated; the maximum activity obtained was 2.95±0.30U/mL at 72h; the economic yield was 9.03 US\$ (8.11€) of transglutaminase obtained per US\$ spent in nutrients [18]. On the other hand, the production of transglutaminase from enzymatic hydrolysates of sorghum was evaluated, the optimal conditions selected were 10 g/L of yeast extract, 2 g/L of corn steep liquor and 13.7 g/L of sodium caseinate, yielding a maximum activity of 1.128 U/mL and a transglutaminase economic yield of 4.144 US\$ (3.72 €) per US\$ spent in nutrients [19]. However, the red sorghum is an economical cereal and an alternative to use as carbon source in culture media.

## 4. Conclusions

The technical and economical optimization gave the following optimal conditions: 2 g/L of DDGS, 2 g/L of yeast extract and 15 g/L of casein. Using enzymatic hydrolysates of sorghum with this nutrient supplementation, a maximum activity of 0.62 U/mL. The plant with sorghum hydrolysates, the annual cost of raw material is estimated at 134,000 US\$, while the other plant's estimated cost is 327,000 US\$. On the other hand, the gross sale result  $12.3 \times 10^6$  US\$ and  $10.8 \times 10^6$  US\$ for plant with hydrolysates and common

media respectively. The production MTGase plant based on a medium with enzymatic hydrolysates showed profitability, reporting a return on investment time of 4 years and a higher income compared to the MTGase production plant using the common medium. This enzyme is a widely used additive in the food industry; thus, the demand may be greater than expected and this would reduce the time to recover the investment. Because sorghum is a cheap raw material that is not very usable and abundant in the state of Tamaulipas, MTGase production based on enzymatic hydrolysate media is a good option to give this crop additional value.

## 5. Acknowledgements

The author Rodríguez-Castillejos GC is grateful for the scholarships awarded by the CONACyT and the IPN for doctoral studies. The authors are grateful for the funding from the Coordinadora de Fundación Produce A.C. and support from SIP Project 20140206.

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