Optimization of the Culture Medium for the Production of Intracellular *β-Galactosidase* from *Kluyveromyces marxianus*

Lamis Yahya Alammarine

Department of Biotechnology, Faculty of Agriculture, Damascus University, syria

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Corresponding Author:
Lamis Yahya Alammarine
E-mail: lalammarine@gmail.com
Bluesky_sbn@gmail.com

KEYWORDS

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ABSTRACT

This paper investigates the optimization of the production of β-Galactosidase using a yeast universal medium containing lactose by *Kluyveromyces marxianus* in shake flask cultures at 35C, 125 rpm and PH=5. Fermentation technology in the shake flask culture was used to investigate the effect the supplements, including (Trace elements, Isopropyl β-D-1-thiogalactopyranoside (IPTG) and thiamin vitamin), had on **β**-Galactosidase enzyme production *by Kluyveromyces marxianus*. The supplements were separately added to the universal medium of yeast containing lactose. Results of the statistical analysis showed that among the different examined media: supplementing the medium with both (1% trace elements and 1% thiamin vitamin) has a significant effect on ß-Gal production at the 5% significance level respectively.

Introduction

Enzymatic hydrolysis of lactose is one of the most important biotechnological processes in the food industry. It is realized by enzyme β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23), which has received special attention (Furlan *et al.*, 2000; Mlichova and Rosenberg, 2006).

The importance of β-Galactosidase is related to its use in milk and milk derivatives to decrease their lactose content, solving the problem of low lactose solubility and its low degree of sweetening. Furthermore, the economic interest in this enzyme is related to its use in food and pharmaceutical industries (Gekas and Lopez-Leivan, 1985; Fonseca *et al*., 2008). ß-Galactosidase can also solve problems associated with whey utilization and disposal. In addition, ß-Galactosidase is used to avoid the problems of lactose intolerance by individuals who are deficient in lactase (Artolozaga *et al*., 1998). New applications for ß-Galactosidase, such as in the production of biologically active galacto-oligosaccharides, have also been reported in the literature (Dagbagli and Goksungur, 2008).

Beta- Galactosidases are the group of enzymes able to cleave β-linked galactose residues from various compounds and are commonly used to cleave lactose into galactose and glucose (Alliet *et al.,* 2007; Juajun, 2009).

They are widely distributed in numerous biological systems, e.g. microorganisms, plants, and animal tissues, with a marked difference in their properties. Among these possibilities, microbial sources offer several advantages, such as easy handling and high production yields, resulting in the decreased price of β-Gal (Santos *et al*., 1998). Commercially, β-Galactosidase is obtained from microorganisms of different genera (Panesar *et al*., 2006). Bacterial and yeast sources are preferable because of ease of fermentation, high activities of enzyme, and good stability. The lactose-fermenting yeasts *Kluyveromyces marxianus* and *Kluyveromyces lactis* are both important industrial yeasts in classical applications with biomass, enzymes and single-cell protein production (Inchaurrondo *et al, 1994;* Rubio-Texeira, 2006). *Kluyveromyces marxianus* offers great advantages, which were used in this study, such as good growth yield, acceptability as a safe microorganism, and higher ß-Gal activity than other yeasts, when lactose is used as a substrate (Belem and Lee, 1998).

The optimization of fermentation conditions, particularly the physical and chemical parameters, is important in the development of fermentation

processes, due to their impact on the economy and practicability of the process (Francis *et al*., 2003). The growth and enzyme production of the organism are strongly influenced by medium composition, thus optimization of media components and cultural parameters is the primary task in a biological process (Dakhmouche *et al.,* 2006).

In order to improve the Beta-Galactosidase production, several groups have made investigations to select microorganisms that have high activity (Thigiel, and Deak, 1989*;* Furlan *et al*., 1995), to evaluate substrates (Grubband Mawson*,1993;* Domingues *et al*., 2004) and to define optimized fermentation conditions for the chosen microorganism (Ramírez Matheus and Rivas, 2003). Several papers have been published (Chen *et al*. 1992; Fiedurek and Szczodrak, 1994; Bojorge *et al*., 1999; Furlan *et al*., 2000; Furlan *et al*., 2001; Dagbagli and Goksungur, 2008; Manera *et al.,* 2008; Pinheiro *et al.,* 2003) reporting the optimization of the production of ß-Galactosidase by *Kluyveromyces marxianus.*

All of the recent papers reported optimized enzyme production through the optimization of fermentation conditions or media components. This current study examined the optimization of ß-Galactosidase production from a yeast universal medium containing lactose by adding supplements and studying their effects on production.

Materials and Methods

(1) Microorganisms

The *DSM 7239 Kluyveromyces marxianus* strain was obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures). The yeast was isolated from yogurt and the risk group for it is 1 classified according to the German TRBA (Technical .Rules for Biological Agents)

(1.1) Media

(1.1.1) *Yeast Universal Medium*:

Table 1: Illustrates the universal medium.

Component	Yeast extracts	Malt extracts	Peptone	Glucose
Concentration (g/l)	3	3	5	10

For ß-Gal production we used lactose and glucose as carbon sources (8:2)

(1.1.2) *Culture Media:*

Three different supplements (trace elements, thiamin vitamin, and IPTG) were separately added to a yeast universal medium containing lactose to discover their effect of product yield; (Table: 2).
*Vitamin: 1% vitamin solution,
*IPTG: 0.5,1,1.5 mM, and
*Trace element.

Table 2: Illustrates trace elements solution components. 1%v was Added.

Chemical	mg/l	Chemical	mg/l
$MnSO_4 \times H_2O$	40	KJ	2
$CoCl_2 \times 6\ H_2O$	16	$NiSO_4 \times 6\ H_2O$	1.8
$AlCl_3 \times 6\ H_2O$	40	$ZnSO_4 \times 7\ H_2O$	4
$KCr(SO_4)_2 \times 12\ H_2O$	4	$Na_2MoO_4 \times H_2O$	8
$CuCl_2 \times 2\ H_2O$	4	$H3BO_3$	2

(1.1.3) *Pre-culture Medium:*
Yeast universal medium was used for preparing pre-culture medium. The components were dissolved in 1L distilled water.

(1.2) Chemicals

(1.2.1) *PM Buffer:*

Table 3: PM buffer components

Component	NaH_2PO_4	Na_2HPO_4	$MgSO_4$	$MnSO_4$
Concentration	0.037 M	0.063 M	1 mM	0.2 mM

(1.2.2) *O-nitrophenol-ß-D-galactopyranoside (ONPG) Solution*:
ONPG solutions were prepared by the addition of solid ONPG into PM buffer solution (0.0133M) (0.040 g ONPG in 10 ml buffer).
(1.2.3) *Saturated solution of* $N_{a2}C_{O3}$ *and B-Galactosidase Standard (10U/ml)*.

(1.3) Fermentation Conditions and Enzyme Production

Fermentations were carried out in 250 ml Erlenmeyer flasks, using 150 ml of culture medium (pH=5), sterilized either by autoclave (121 C, 15 min) (all universal medium components except

glucose and lactose were separated autoclaving) or by filtration (vitamin and trace elements). The flasks were inoculated with the pre-culture volume (10%). The cultures were incubated at 35ºC for 48 hours in a rotary shaker incubator at a shaking speed of 125 rpm. The pH medium was adjusted to 5, either1M NaOH or 1M HCl. In the optimization, thiamine vitamin and trace elements were examined separately and together and supplemented into the culture media to investigate their effect on the ß-Gal production by *Kluyveromyces marxianus*. The effect of IPTG was also investigated by adding three various concentrations (0.5, 1, and 1.5) mM to the media. The yeast was grown in shake flasks in those various tested media, Table (4).

Table 4: Demonstrates the different examined media

Medium	Universal medium; containing lactose ;components (g/l)					Supplement		
	Peptone	Yeast extract	Malt extract	Lactose	Glucose	Thiamin 1%	Trace elements	IPTG mM
1	5	3	5	8	2	0	0	0.5
2	5	3	5	8	2	0	0	1
3	5	3	5	8	2	0	0	1.5
4	5	3	5	8	2	1%	0	0
5	5	3	5	8	2	0	1%	0
6	5	3	5	8	2	1%	1%	0

(1.4) OD Measuring

Turbidity is measured with a spectrophotometer, an instrument that passes light through a cell suspension and detects the unscattered light that emerges. The unit of turbidity measurement is optical density (OD) at the wavelength specified (Madigan *et al*, 2008). Growth of the yeast cells through fermentation were determined from measurements of the optical density (O_{D600}) at a wavelength of 600 nm carried out by means of a *Pharmacia Biotech Novaspec II* spectrophotometer. The samples were collected from the flask in 10mm couvettes and the OD was measured at 600 nm after using sterile water as a blank. A standard calibration chart was used within a linear range of (0- 0.7) OD units. Samples with an OD greater than 0.7 units were diluted so that the OD was within the calibration linear range:

$$OD_{total} = OD_{measured} \times DF \text{ (Dilution Factor)}.$$

(1.5) Megerments of Glucose and Lactose Substrates

Lactose and glucose concentrations through fermentation were determined by means of an *YSI biosensor Model 2700 Select*. The principle is that an enzyme specific for the substrate of interest is immobilized between two membrane layers: polycarbonate and cellulose acetate. The substrate is oxidized as it enters the enzyme layer, producing hydrogen peroxide, which passes through cellulose acetate to a platinum electrode, where hydrogen peroxide is oxidized. The resulting current is proportional to the concentration of the substrate. YSI membranes contain three layers. The first layer, porous polycarbonate, limits the diffusion of the substrate into the second enzyme lone, preventing the reaction from becoming enzyme limited. The third one, cellulose acetate, permits only small molecules, such as hydrogen peroxide, to reach the electrode, eliminating many electrochemically-active compounds that could interfere with measurement (Figure 1), (see, www.ysilifesciences.com.com).

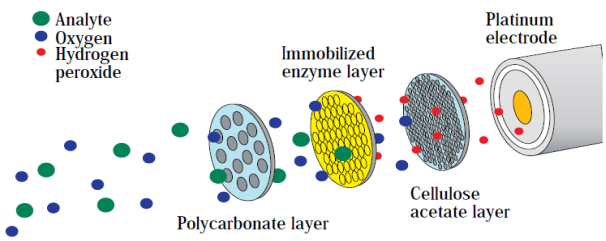

Figure 1: The reaction that takes place in aYSI immobilized-enzyme biosensor.

(1.6) Product: Beta-Galactosidase Assay

ß-Gal can be assayed by measuring hydrolysis of the chromogenic substrate, ONPG, as shown below (Miller, 1972). When the ß-Gal cleaves ONPG, o-nitrophenol is released. The amount of o-nitrophenol formed can be measured by determining the absorbance at 420 nm. If excess ONPG

is added, the amount of o-nitrophenol produced is proportional to the amount of ß-Galactosidase and the time of the reaction. The reaction is stopped by adding Na_2CO_3 which shifts the reaction mixture to pH 11. At this pH, most of the o-nitrophenol is converted to the yellow-colored anionic form, and ß-Galactosidase is deactivated.

Analytical Method

Two independent experiments were carried out for the growth of DSM *7239-Kluyveromyces marxianus* strain in each medium. The effects of the supplementing substances (IPTG, trace elements, and thiamin) on ß-Gal enzyme production were analyzed according to statistical analysis program (SPSS V19).

Results and Discussion

(1) Influence of IPTG

Figure (2) shows the optical density (OD_{600}) of *DSM 7239-Kl.marxianus* for the culture media 1, 2, 3 during the fermentation process. There is not much difference between the growth of yeast (6.3, 6.32, 6.5) on the three different media 1, 2, 3 supplemented with 0.5, 1, 1.5 mM IPTG. The statistical analysis also showed no significant effect of supplementing IPTG on the *Kl. marxianus* growth. Analysis of variance is presented in Table (5).

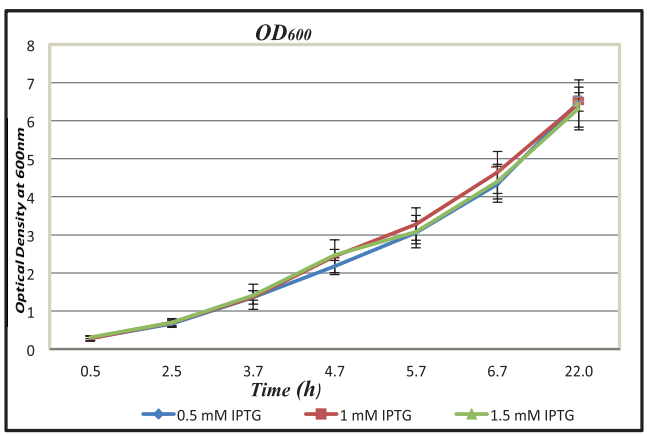

Figure 2: Growth of DSM 7239-Kluyveromyces Marxianus in 3 Different Culture Media 1, 2, 3 Supplemented with IPTG in Shake Flask. 3

Figure (3) shows the production of Beta Galactosidase from *DSM 7239 – Kl. marxianus* for the culture media 1, 2, 3 during the fermentation process. The results have shown that the ß-gal activity (U/ml) was (2.4, 3.5, 2.8) for the supplemented medium with 0.5, 1, 1.5 mM IPTG. Analysis of variance (ANOVA) for enzyme activity is presented in Table (6) showed that there is not any significant effect of IPTG on the enzyme production at the 5% level (p> 5%)

Table 5: ANOVA for optical density

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	0.662	03	0.221	0.049	0.985
Within Groups	202.859	45	4.508		
Total	203.521	48			

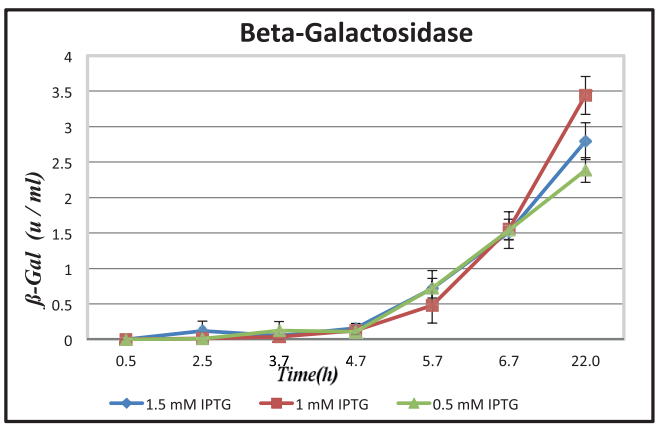

Figure 3: *ß-Galactosidase* (u/ml) produced by DSM 7239 *Kluyveromyces marxianus* for the culture media 1, 2, 3 supplemented with IPTG in Shake Flask.

Table 6: (ANOVA) for *ß.Galactoside* production

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	01.767	04	0.442	0.436	0.782
Within Groups	44.560	44	1.013		
Total	46.327	48			

(2) Thiamin Vitamin, Trace Elements, Thiamin and Trace Element Influence

Figures (4) & (5) show the OD $_{600\ of}$ *DSM 7239 - Kluyveromyces marxianus* and their production of ß-Gal for the culture media 4, 5, 6 during the fermentation process. Media 4, 5, 6 were respectively supplemented with thiamin vitamin solutions 1%., trace elements 1 %., with both thiamin and trace elements.

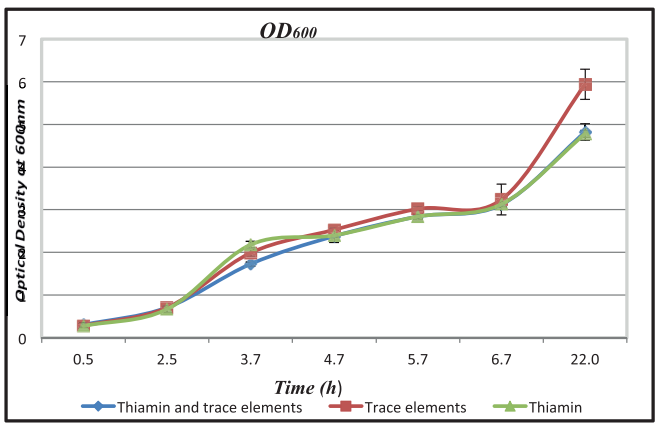

Figure 4: Growth of DSM 7239 *Kluyveromyces marxianus* on three different culture media 4, 5, 6 supplemented with thiamin, trace elements, and both in Shake Flask.

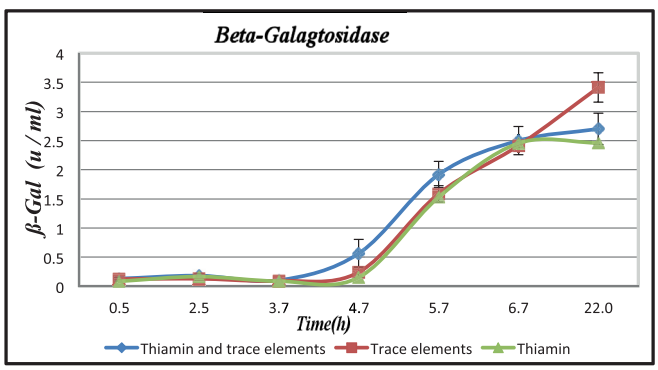

Figure 5: (ANOVA) for *ß.Galactoside* production

The schemes show that the medium with the vitamin had OD = 4.7, ß-gal activity= 2.5 U/mL and OD= 5.9 and *ß.Galactoside* activity= 3.4 U/mL for the medium with trace elements, whereas the OD=4.8 and ß-gal activity=2.7 U/mL for the medium which supplemented with both trace and thiamin vitamin. The statistical analysis, tables (7), showed that there is no significant effect for supplementing the thiamin vitamin or trace elements, whereas there is significant effect for supplementing with both vitamin and trace element to the yeast universal medium containing lactose at the 5% level of significance respectively. According to the Table (9), p value=0.037= 3.7% < 5%

Table 7: Independent samples test for vitamin, for trace elements, and for element and vitamin

		Levene's Test for Equality of Variances		t-test for Equality of Means					
		F	Sig.	t	df	Sig. (2- tailed)	Mean Difference	Std. error difference	Confidence 95% Interval of the Difference
									Upper
									Lower
ß.Gal Equal variances assumed, Equal variances not assumed	(*) ☑	16,388	,001	1,452	19	,163	,64148	,44169	1,56596 1,37031
	☒			1,843	18,889	,081	,64148	,34808	-,28300 -,08735
	(**) ☑	12,634	,002	1,524	19	,144	,78926	,51779	1,87301 1,62043
	☒			1,994	18,173	,061	,78926	,39589	-,29448 -,04191
	(***) ☑	20,775	,000	1,763	19	,094	,79711	45214,	1,74345 1,53969
	☒			2,248	18,818	,037	,79711	35455,	-,14922 ,05454

(*) *Independent Samples Test for Vitamin*
(**) *Independent Samples Test for Trace Elements*
(***) *Independent Samples Test for Trace Elements and Vitamin*
☑ *ß.Galactoside Equal variances assumed*
☒ *ß.Galactoside Equal variances not assumed*

Concluding Remarks

In this study, a *ß.Galactoside* enzyme was produced by DSM *7239- Kl.marxianus* using yeast universal medium containing lactose. Trace elements 1%, IPTG (0.5, 1, and 1.5 Mm) and thiamin vitamin1% were supplemented to the culture

medium to investigate their effect on the enzyme production. The statistical analysis have shown that IPTG at the three different concentrations has no significant difference on the Beta Gal production which has been reported by (Avinash, *et al.,* 2002; Donovan, *et al.,*1996). This study also found no significant effect for supplementing the vitamin 1% and trace elements1% on the Beta Galactosidase production. Whereas this work was found, a significant difference were obtained for ß-Galactosidase activity, when the universal yeast medium containing lactose supplemented with both trace element 1% and thiamin vitamin 1%.

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