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Effect of Agitation on Taq DNA Polymerase Production by *Escherichia Coli* in Bioreactor

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The Taq DNA polymerase is an important protein used during PCR (Polymerase chain reaction), a technique widely used today. The production of this enzyme in bioreactors make possible to improve efficiency, to reduce production time, to increase production and expression, or to reduce production costs. This is achieved thanks to the measurement, monitoring and control of the different parameters that influence this biotechnological process: dissolved oxygen, pH, temperature, incubation time, agitation during the incubation and induction phase.In the present work, the production of Taq polymerase expressed in *Escherichia coli*, was made on a bioreactor scale, BioFlo/CelliGen 115®, with a volume of 1 L, equipped with temperature (37 °C) and pH (6.5) control, and with a constant level of dissolved oxygen provided by a 1 vvm flow of air. After the induction phase, we extracted the polymerase by cell disruption using centrifugation and some substances. For the protein measurement was implemented d technique and SDS-PAGE electrophoresis.
According to gel electrophoresis results, molecular weight of the protein obtained in bioreactor was 80 kD, similar to the value of commercial production in flasks. The effect of agitation on protein concentration after induction was studied at two levels: 220 and 350 rpm, the study found a production of 1465 $\mu g\ L^{-1}$ and 2975 $\mu g\ L^{-1}$ of protein respectively. The results confirm a higher production of protein in this bioreactor, keeping the type of polymerase. This could be the first step to scale the production with controlled conditions at higher rates.

1. Introduction

The production of heterologous proteins on a bank and industrial scale represents the opportunity to design bioprocess with higher yields and profitability that allow more accessible commercial values, especially in the field of industrial biotechnology in Colombia. The Taq DNA polymerase is an essential enzyme used to amplify DNA sequences by PCR technique, the enzyme was extracted from thermophilic bacteria and for its production it is expressed in a fast-growing bacteria *Escherichia coli* as heterologous protein (Sung-Gun and Jong-Tae, 2014).

Several factors affect the expression of a metabolite of interest on the part of a microorganism, aspects such as metabolism, genetic regulation, and the conditions of bioreactor operation, it is important to consider criteria such as volume, temperature, aeration, and agitation of the system, among others. In the case of products obtained by recombinant DNA technology, it is necessary to specify how these conditions affect the stability of the plasmid reflected in the expression of the protein (Che et al., 2016).
The production of Taq Polymerase is documented at laboratory scale in flasks; however, this limits the options to constantly monitor and control the evolution of the fermentation process. Bioreactors allow the development of different biotechnological processes because they offer operational control at different scales (Liu.,2020). The bioreactor used in this work is the BioFlo/Celligen 115®, a continuous agitation bioreactor, which provides process control for pH, agitation, dissolved oxygen and temperature, and in turn has the Biocommand software, which allows monitoring environmental parameters (Eppendorf., 2009).

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The main objective of this work was to study the effect of agitation on the production of the commercial enzyme Taq polymerase Tucan Taq® from a recombinant strain of *E. coli* in a bioreactor with an effective volume of 400 mL, keeping an airflow of 1 vvm. The results of the enzymatic expression were compared with the laboratory-scale production in shake flasks to establish that the production on a larger scale does not alter the stability of the plasmid and the bioproduct.

2. Materials and method

2.1 Microorganism

E. coli strain DH5α was transformed with a plasmid which contains a Taq polymerase gene. Transformation and activation were performed in broth LB (10 g/L triptone, 5 g/L yeast extract, 10 g/L sodium chloride) (Merck, 2020) supplemented with ampicillin 100 μg/ml. The culture was incubated at 37 °C for 12 hours.

2.2 Preinoculum

Inoculum was inoculated in a shake flask with 200 mL of LB-ampicillim broth for transformed strain. Culture was incubated at 37 °C at 200 rpm in a shaker incubator.

2.3 Batch Fermentation Condition and expression plasmid

The conditions of bioreactor, BioFlo/Celligen 115®, was as follows: presterilized 400 mL LB-ampicillim broth (working volume), 1% of preinoculum, temperature 37 °C, airflow rate 1 vvm (volume of air per unit of growth medium per minute). Two agitation rates (200 -350 rpm) were evaluated. The batch fermentation process continued until obtain an optical density ($OD_{595\ nm}$) between 0.28 – 0.32. The induction was performed by 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and continued for 16 hours (Singh et al., 2015). The experiments were performed by duplicate for two agitation speeds.

2.4 Biomass determination

Biomass was determined by optical density at 595 nm ($OD_{595\ nm}$), from aliquots of fermentation broth, was collected at every 60 minutes during the first 9 hours of fermentation process (before and after induction stage).

2.5 Extraction and purification of Taq Polymerase

The fermented broth was recovered in centrifuge tubes, they were spun at 4800 rpm during 20 minutes at 4°C. The fermented cell washed pellet was suspended with 30 mL pre-cell lysis buffer (50 mM Tris-HCl, 50 mM dextrose, 1 mM EDTA and 100 μg/mL of lysozyme) and agitated by vortex (Ferralli et al., 2007). The supernatant was incubated at 37 °C for 30 minutes and suspended in cell lysis buffer (50 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 1 % Triton x-100 and PMSF) (Farazmandfar et al, 2013) it was agitated by vortex and incubated in a water bath at 75°C for an hour. Protein was obtained by centrifuging at 10000 rpm for 15 minutes at 4 °C.

2.6 Protein determination and quantification

Protein concentration was determined according to Bradford (Bradford, 1976) using gammaglobuline at 640 μg/mL as patron curve. Coomassie blue G-250 was used for protein visualization. Gel electrophoresis was used to analyze protein (Biorad, 2020) and visualization was made by Coomassie blue and a molecular weight tracer with a range of 11 kDa to 245 kDa. Each agitation level was measured by duplicate.

2.7 Measurement of operation parameters

Dissolved oxygen and pH were monitored by means of software Biocommand ® and the respective electrodes for all the fermentation process (21 hours), giving 21 data or each replica in each of the agitation level.

3. Results and Discussion

3.1 Biomass determination

The indirect measurement of cell growth by optical density was performed during the first 9 hours. In the first 4 hours occurs latent phase and the following hours the exponential phase. About the fifth hour of incubation in which the OD was in the range of 0.28 - 0.32, the induction process was carried out with 0.5 mM of IPTG. Figures 1 show that cell growth is similar at both levels of agitation, however, in the agitation at 350 rpm there was a slight increase over the last few hours evaluate. The monitoring of microbial growth and its physiology allow to infer that the cells did not present stress when changing from an orbital shaking agitation to a continued stirred reactor, this system may favour oxygen mixing and transfer processes which may influence cell integrity

and increase in protein production (Bagagli and Sato 2013). These results can be a first approximation, considering that the relation between aeration rate and impeller agitation speed is so important as a scale-up parameter in the protein production by *E.coli* as a host microorganism (Espinosa et al., 2018).

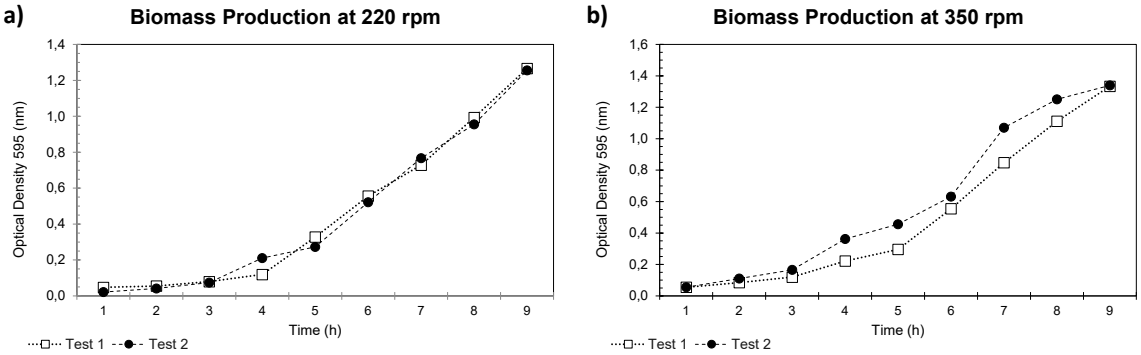

Figure 1: Biomass analysis at different agitation speeds a) 220 rpm, b) 350 rpm

3.2 Protein determination and quantification

As Table 1 shows, at 350 rpm there is a higher production of protein than at 220 rpm, showing that the aeration and mixing process is more effective, managing to better supply the respiratory and energy demand required for the metabolic processes of *E. coli*, biosynthetic processes require large dell energy investments therefore oxidative metabolism should not be limited to supply these needs. Tan et al., (2018) analyzed the influence of agitation speed on production of a protein with a recombinant *E.coli DE3,* they observed the highest growth and production were at an agitation speed of 1000 rpm compared whit less speeds.

Table 1: Protein concentration

Agitation (rpm)	Test	NIC[1] (μg/mL)	ANIC[2] (μg/mL)	IC[3] (μg/mL)	AIC[4] (μg/mL)
220	1	857	883.25	1352.66	1465.83
	2	909.5		1579	
350	1	1141	1197.75	3071	2973.5
	2	1254.5		2876	

[1]Non induced concentration; [2]Average Non induced concentration; [3] Induced concentrations; [4] Average Induced concentration

Experimental production of the Tucan Taq® was carried out at 210rpm with orbital agitation with an average final enzyme production of 1633 μg/mL (data provided by CorpoGen). In relation to the results in Table 1 and Table 2, bioreactor production with a mechanical stirring system at 350 rpm improved by 82% compared to flask-level production. However, at an agitation speed of 250 rpm the production was lower, which may imply deficiencies in the transfer of oxygen and mass within the system. Higher efficient oxygen transfer includes an improvement in the yields of biomass product, especially when talking about the expression of metabolites associated with growth (Falco et al., 2014).

Table 2: Protein production at Flask and Bioreactor level

Scale	Agitation (rpm)	APE[1] (μg/mL)	PIP[2] (μg/mL)
Flask	210 (orbital)	1633	-
Bioreactor	350	2973.5	82.08
	220	1465.8	-10.23

[1]Average Production Enzyme; [2]Percentage Improvement Production compared whit flask level

It is important to clarify that the experimental processes at flask and bioreactor scale were carried out under similar conditions such as working volume (400ml), temperature, culture media and pH, so a decrease in the production of Taq polymerase at the lowest speed in bioreactor suggests an effect on enzyme expression; however, this requires a greater number of experiments to infer that.

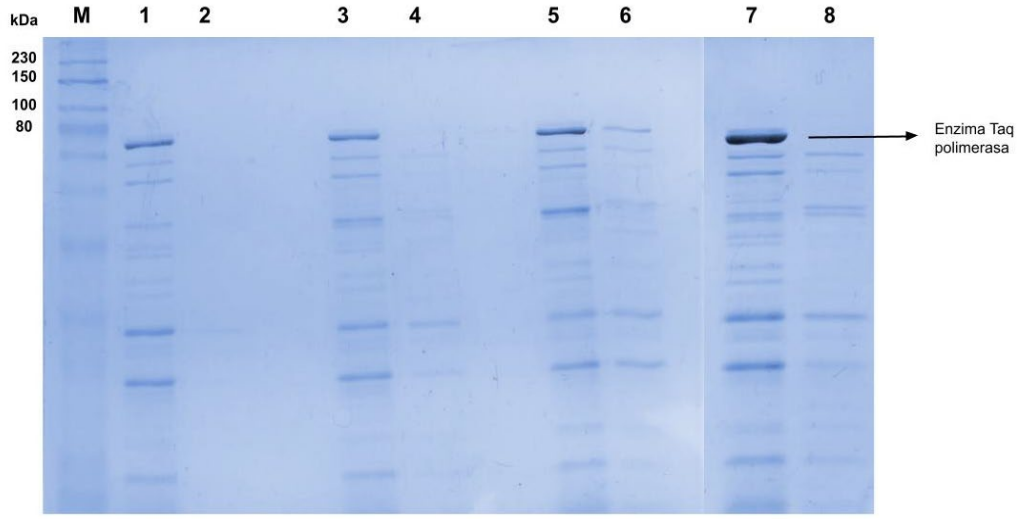

Figure 2. Polyacrylamide gel electrophoresis for the expression and purification of Taq polymerase from recombinant E. coli Tucan Taq® . Lane M, protein molecular weight marker; Lane 1, E. coli after inducing, test 1 at 350 rpm; Lane 2, E. coli before induction, test 1 at 350 rpm; Lane 3, E. coli after inducing, test 2 at 350 rpm; Lane 4, E. coli before inducing, test 2 at 350 rpm; Lane 5, E. coli after inducing, test 2 at 220 rpm; Lane 6, E. coli before inducing, test 2 at 220 rpm; Lane 7, E. coli after inducing, test 1 at 220 rpm; Lane 8, E. coli before inducing, test 1 at 220 rpm.

In comparison with the samples before induction, the absence of the expected band in the molecular weight of Taq polymerase is evidenced in Figure 2, indicating that these samples do not contain the protein of interest. For all the replicates of each level of agitation, it is observed that the samples after induction present an intense band at a molecular weight of approximately 80 kDa, indicating the success of the induction and expression of the enzyme, since the presence of Taq is evident in the different samples. Although the Bradford technique shows an increase in protein concentration at 350 rpm, the gel electrophoresis technique only allows inferring whether the induction was successful or not.

3.3 Measurement of operation parameters

A change in agitation speed does not generate a significant difference in the measured pH values (Figure 3); in addition, they are in the optimal range for growth of *E. coli*, this being 6 - 8.6. The pH was not controlled by adding acids and bases to the fermentation, but it was monitored to ensure that the host and the enzyme of interest are found in a growth medium that will generate a neutral pH range. According to different studies of Taq polymerase production, the reported pH is the one provided by the culture medium used, being the LB, with a pH of 6.8 to 7.2, it should be clarified that this production was carried out in a flask (Farazmandfar et al., 2013). The effect of agitation on the availability of dissolved oxygen (Figure 4) in the medium is significant, generating a decrease in this element for 5 hours when the agitation was at 220 rpm compared to 350 rpm where the decrease was for 1 hour. At lower levels of agitation, cells need more time to be able to adapt their metabolism to the metabolic load imposed by all the biological processes carried out (Sørensen and Mortensen, 2005). At 350 rpm, being a higher agitation speed, a bubble size smaller than 220 rpm is generated and it gives a greater contact area and therefore the diffusion of dissolved oxygen in the medium is greater (Kulkarni et al., 2009).

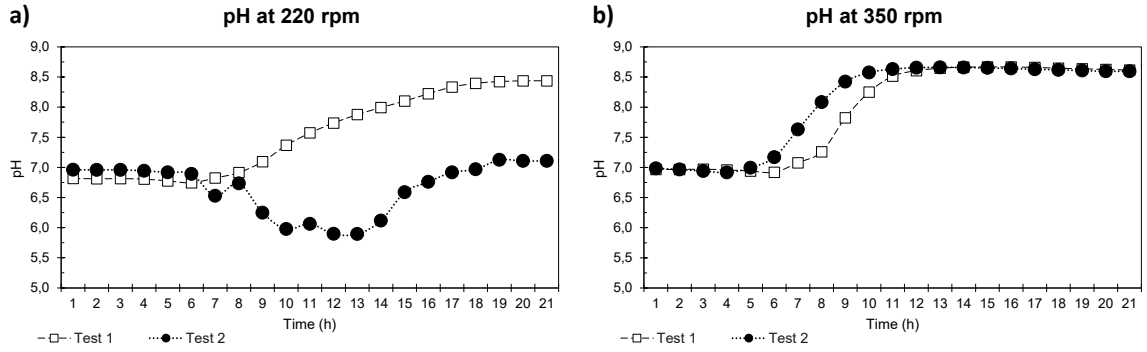

Figure 3: pH behavior at different agitation speeds a) 220 rpm, b) 350 rpm.

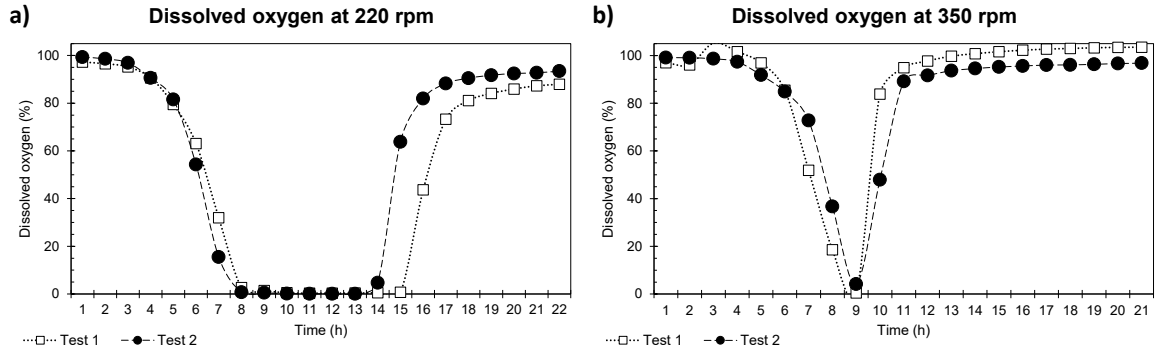

Figure 4*:* Dissolved oxygen behaviour at different agitation speeds a) 220 rpm, b) 350 rpm

4. Conclusions

Continuous mechanical agitation of the bioreactor influences greatly on the concentration of the recombinant protein of Taq polymerase expressed in *Escherichia coli*. It increases the concentration at higher levels of agitation, on average 82% in the level of 350 rpm compared to flask scale. Production of Taq polymerase at a bioreactor scale is of great importance since the demand for the PCR technique in the world has increased considerably. Finally, bioreactors for production of recombinant proteins have easier monitoring and control of the different environmental parameters providing advantages when it comes to an industrial process.

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