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Aeration Rate Effect on the Growth Kinetics, Phytase Production and Plasmid Stability of Recombinant *Escherichia coli* BL21 (DE3)

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Phytase production using *Escherichia coli* BL21 (DE3), a recombinant strain harboring a plasmid encoding thermostable *Bacillus* phytase, in semi-industrial scale was studied in this work. Among the factors needed to be considered in order to achieve high enzyme yield, aeration rate plays an important role. Suitable aeration is required to supply cells with sufficient amount of air for cell and phytase production. The effect of different aeration rates, (1.0, 2.0 and 3.0 $vv^{-1}min^{-1}$), on the kinetics of cell growth and phytase production by the recombinant *E. coli* BL21(DE3) in 16-L pilot scale stirred tank bioreactor was investigated. The highest cell concentration of 3.81 gL^{-1} concomitant with the maximal total phytase production of 15.63 UmL^{-1} were obtained in a bioreactor of aeration rate 3.0$vv^{-1}min^{-1}$. At this high aeration rate, a maximal specific growth rate (μ_{max}) and the maximal specific phytase production of 0.33 h^{-1} and 4102Ug^{-1}, respectively, were achieved.

Key words: Phytase production, *Escherichia coli*, aeration rate, Plasmid stability, bioreactor cultivation.

The production of microbial industrial enzymes has recently drawn attention in the biotechnology of animal feeds[1]. Nowadays, phytase has become a very important microbial enzyme, which is applied as feed additive for monogastric livestock. Phytase hydrolyses phytate (myo-inositol-1,2,3,4,5,6-hexakisphosphate), which is the main form for storing phosphorus in most of the seeds and grains used as animal feed components[2,3]. Animals cannot hydrolyze phytate by themselves, and hence the application of phytase enzyme results in the liberation of phosphate groups which then becomes available for animal utilization. Otherwise, organic phosphate has to be added to meet certain level required in feed content. Recombinant *Escherichia coli* has been widely applied as a bacterial platform for expressing simple heterologous proteins such as phytase[4-8]. Since most of the phytase in recombinant *E. coli* is expressed extracellularly, the productivity of phytase is relativeto the final cell density and the specific productivity[9]. However, under normal cultivation condition s,*E. coli* can only express low amount of phytase[10]. Generally,

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the major objectives of most processes invlove recombinant *E. coli* is to achieve high biomass production and to improve the volumetric productivity. One of the key factors controlling such process is the air supply. *E. coli* is a facultative anaerobic bacterium which is capable to live either in the presence or absence of oxygen[11]. In order to achieve high recombinant phytase production through increasing the biomass, a high capacity of oxygen supply is required, which is generally controlled by agitation and/or aeration rate[12]. It has been previously reported that suitable air supply in bioreactor culture will improve the cultivation performance of *E. coli* and accordingly, the production of phytase enzyme. The aim of the present investigation was to evaluate the effect of applying different aeration rates on the growth kinetics, phytase production and plasmid stability of the recombinant *E. coli* BL21(DE3).

MATERIALS AND METHODS

Microorganisms

The phytase producer recombinant strain, *E. coli* BL21 (DE3) used in the present study was kindly provided by Prof. Dr. RajniHatti-Kaul, Biotechnology Department, Lund University, Sweden. This strain harbors pE10C2 plasmid which include a thermostable *Bacillus sp.* phytase gene[13]. Cells were first propagated on LB agar medium, incubated at 37ºC for 24 hours. The arisen colonies were harvested in 50% glycerol solution and maintained in 2 mL cryogenic vials(Nalgen, USA) at -80°C as cell bank in WICC (Wellness Industries Culture Collection, Institute of Bioproducts Development, Universiti Teknologi Malaysia, Johor Bahru, Malaysia) for subsequent use. These tubes were frozen immediately at -20ºC for 24 hours followed by further storage as working cell bank at -80ºC for further use. The viability of the deep-frozen cultures was periodically controlled by plating onto LB agar medium.

Inoculum preparation

For each experiment, 1 cryogenic vial from the working cell bank was thawed and used to inoculate 50 mL LB broth in 250 mL Erlenmeyer flask. The inoculated flasks were incubated on rotary shaker at 200 rpm and 37ºC for 24 hours. The grown cells were used to inoculate either shake flask or bioreactor cultures in concentration of 1% vv^{-1} using bacterial suspension of OD_{600}=1.

Medium for cell mass production

For aeration study, the optimized medium used was composed of [gL^{-1}]: glucose, 15; $(NH_4)_2SO_4$,4; $(NH_4)_2HC_6H_5O_7$, 1; K_2HPO_4, 6.5; $NaH_2PO_4.H_2O$,1.541; $MgSO_4.H_2O$,1.44;2 mL trace elements solution and a mixture of 30 mM lactose and 10 mM $CaCl_2$ as an inducer. The pH was adjusted to 7.0 before sterilization. Glucose was sterilized separately and added to medium before inoculation.

Cultivation conditions

Inoculum was prepared in 250 mL Erlenmeyer flask containing 50 mL liquid LB medium. After 24 hours incubation, the vegetative cells were used to inoculate the production medium to give a final cell concentration of 0.1 $OD_{600.}$ The inoculated flasks were incubated at 37ºC on rotary shaker at 200 rpm for 24 hours. For bioreactor experiments, cultivations were performed in 16-L pilot scale *in situ* sterilizable stirred tank bioreactor (BioEngineering, Wald, Switzerland) with a working volume of 6-L. Two bioreactors were run in parallel at aeration levels of 1.0, 2.0 and 3.0 $vv^{-1}min^{-1}$. Temperature and agitation speed were adjusted at 37ºC and 200 rpm, respectively. The aeration supply was performed using sterile air and the aeration rate was controlled by integrated thermal mass flow controller. The pH was determined throughout the cultivation using *in situ* sterilizable pH electrode (Mettler-Toledo, Urdorf, Switzerland) attached to the control panel of the bioreactor. The cultivation of *E. coli* BL21(DE3) was run at the neutral pH, where the pH was controlled and adjusted by the automatic addition of 1M NaOH solution. The dissolved oxygen concentration (DO) was analyzed using *in situ* polarographic electrode (Ingold, Mettler-Toledo, Urdorf, Switzerland). During cultivation, foam was suppressed by the addition of the antifoam agent Struktul (Th. Goldschmidt AG, Essen, Germany).

Sample preparation, cell dry weight determination and plasmid stability

Samples were collected every 2 hours from the bioreactor (about 30.0 mL) in pre-weighed 50 mL sterile centrifugation tube (Falcon, USA). The OD of sample was measured immediately at 600nm using spectrophotometer. Two ml. of sample was taken for plasmid stability using two different agar media (LB agar and LB plus ampicillin) using

spread plate technique. Cells in the rest of sample were then separated using cooling centrifuge at 5000 RPM and 4 °C for 20 min. Cell pellet were kept frozen at -20 °C and used thereafter for the determination enzyme activity. The supernatant was also frozen immediately for other biochemical analysis (extracellular enzyme activity and glucose).

Glucose analysis

The supernatant was diluted with distilled water with a dilution factor of 1:10.1 mL sample was then pipetted into a triplicate set of test tubes and 1.0 mL 3,5-dinitrosalicylic acid (DNS) reagent was added into each test tube for determination of residual glucose by DNS method[14]. The samples were then incubated at 90ºC for 15 min, ice-cooled, followed by addition of 8.0 mL deionized water. The intensity of the developed brown color was read at 540 nm. Glucose concentration of the sample was then determined according to the previously plotted standard curve.

Analysis of extracellular, periplasmic, cytoplasmic (intracellular) phytases

For phytase analysis, the sample was first centrifuged at 5000 rpm at 5 °C for 10 min. The supernatant was collected for extracellular analysis while the pellet was washed with 3mL of saline solution, mixed gently and was centrifuged again. The supernatant was discarded and the pellet was dissolved in 3.0 mL solution A (30 mM Tris HCl, pH 8, 20 % sucrose (w/v), 5 mM $CaCl_2$, EDTA), and gently mixed followed by second round of centrifugation. The obtained cell pellet was mixed with 5.0 mL cold $MgSO_4.H_2O$ and hold in ice for 10 min. For analysis of periplasmic phytase activity, the sample was centrifuged and the phytase activity was determined in the supernatant. The pellet was used to determine cytoplasmic phytase content. Five mL 0.1 M Tris HCl, pH 7 was added to the sample followed by sonication for 2 minutes using 20 seconds cycle intervals. After sonication, the sample was centrifuged again and supernatant was collected for the determination of cytoplasmic phytase. For all sample preparations, phytase activity was determined base on the colorimetrical quantification at 700nm of free phosphorus released by the hydrolysis of phytate using ammonium molybdate as color reagent[15]. The enzyme sample was first diluted and mixed with phytate solution which composed of 1.5 mM sodium phytate dissolved in 100 mM Tris-HCl buffer (pH 7) containing 5 mM $CaCl_2$. The mixture was then incubated at 70 °C for 20 min and stopped thereafter by addition of an equal volume of 15% tricholoacetic acid solution. The precipitate was separated by centrifugation and 500 ml of the supernatant was mixed with molybdate reagent (containing 1.4 mixture of 2.7% $FeSO_4$ and 1.5% ammonium molybdate in 5.5% H_2SO_4) and incubated for 5 min. before measuring the absorbance at 700 nm. The concentration of the liberated inorganic phosphate (P_i) from sodium phytate was determined based on a previously prepared standard curve using NaH_2PO_4. One unit of phytase activity is defined as the amount of enzyme needed to release 1 mmole of P_i per minute.

Acetic acid analysis

Samples in form of 2.0 mL each were filtered using 0.45 µm sterilized filter to remove any possible biomass residues before analysis. Acetate was assayed according to the method of Pecina *et al.* (1984)[16] which was modified later by Tomlins *et al.*(1990)[17] using HPLC system (Waters, Milford, MA, USA). This system composed of a pump Waters 600 controller, 2690 Separation Module HPLC and auto sampler fitted with a detection system; Ultraviolet (UV) 2487 Dual Absorbance Detector at 210 nm. Separation was performed using organic acid column: 300×7.8 mm/ 8 micron (Phenomenex, Torrance, USA). Acetic acid was eluted with 0.005N sulphuric acid at a flow rate of 0.5 ml/min at 40 °C. Peak heights were measured using a dual channel computer integrator (Water Empower chromatography system, Waters, Milford, MA, USA) and converted to acetic acid concentration based on standard solution.

RESULTS AND DISCUSSION

Profile of different growth parameters (glucose concentration, cell dry weight and DO) as affected by different aeration rates

Parallel fermentation experiments were performed in 6-L working volume operated stirred-tank bioreactor at the three different aeration rates investigated; $1.0vv^{-1}min^{-1}$ (Fig.1), $2.0vv^{-1}min^{-1}$ (Fig. 2) and $3.0vv^{-1}min^{-1}$ (Fig. 3). All cultures were induced with lactose solutions after 5 hours of cultivation, when the glucose concentration started to decrease gradually. At that point, glucose

concentration reached 7.61, 7.83 and 9.22 gL^{-1} for the 1.0, 2.0 and 3.0$vv^{-1}min^{-1}$ cultivations, respectively. Furthermore, all cultivations started to enter the log phase, and the cell growth increased exponentially accompanied by a concomitant decrease in the glucose concentration.

The obtained results showed that cell growth increased more rapidly at 2.0 $vv^{-1}min^{-1}$ ($m=0.50\ h^{-1}$) compared to only ($m=0.33\ h^{-1}$) in case of 3.0v $v^{-1}min^{-1}$ aerated culture. The cell growth reached its maximal value (X_{max}) of 3.43 g L^{-1} after 9 hours of cultivation. Under cultivation of 1.0$vv^{-1}min^{-1}$, the growth profile as shown in Fig. 1. From this profile, cells grew rapidly in the exponential growth with specific growth rate of 0.12 $h^{-1}$for about 16 hours. On the other hand, the maximal cell dry weight obtained at 3.0 v $v^{-1}min^{-1}$ reached 3.82 gL^{-1} at 17 hours with a maximal specific growth rate of 0.33 h^{-1}. Generally, maintaining high specific growth rate to reach high biomass is a common strategy, which is mostly adopted by many researchers to improve recombinant protein production. This helps sustaining the optimal cellular health, which is required to overcome the metabolic stress associated with recombinant protein expression[18]. Cultivation at the aeration rate of 1.0 v $v^{-1}min^{-1}$ shown that after 4 hours of inoculation the dissolved oxygen fell rapidly and the cell growth under zero percent of dissolve oxygen for almost 6 hours before induced with lactose. However, the cultivation run at 2.0 v $v^{-1}min^{-1}$ the dissolved oxygen started to decrease from 100 to 0.0% after 6 hours of cultivation, while at 3.0$vv^{-1}min^{-1}$ the DO started to decrease 2 hours later. The fast decrease in the DO indicated the start of the log phase, as the cells started rapidly to grow and use available dissolved oxygen.

Acetic acid is a major obstacle that faces *E. coli* fermentation. Acetic acid is produced by *E. coli* under oxygen limited or anaerobic conditions[11]. In all cultivation, the acetic acid concentration increased over the cultivation as a by-product of the fermentation. High concentration of acetate reduces the growth rate of the culture and lead to low protein expression Therefore due to limitation of dissolved oxygen when cultivated with 1.0 v $v^{-1}min^{-1}$ concomitantly causes accumulation of acetic acid in the fermentation broth almost 4.06 g L^{-1}after 16 hours of cultivation. During cultivation at 2.0 v $v^{-1}min^{-1}$, the maximal concentration of acetic acid (2.1 gL^{-1}) was reached after 23 hours. This value was almost the same as reached in 3.0 v $v^{-1}min^{-1}$ aerated culture. From the data represented in Figs. 1-3, it could be observed that acetic acid started to accumulate directly after the induction phase. These results are in agreement with those reported previously, where it was concluded that high acetate concentrations decreases the growth rate of *E. coli*, thus leading to low protein expression[19-22].

Effect of different aeration rates on the production of phytase

Since the cells were not yet induced to express the phytase enzyme, there was no production during the pre-induction phase. After the induction with a mixture of 30 mM of lactose and 10 mM $CaCl_2$, the cells started to release the phytase enzyme, whose activity was detected at the extracellular, periplasmic and cytoplasmic cell levels (Fig. 1-3). The total activity of phytase was taken as the sum of phytases produced extracellularly, periplasmic and cytoplasmic. The lowest total phytase activity was observed when aeration rate at 1.0 v $v^{-1}min^{-1}$ only 2.52 U mL^{-1} after 4 hours lactose induction. A maximal total activity

Table 1. The growth and production parameters during phytase production by recombinant *E. coli*BL21(DE3) cultivation in 16-L bioreactor with different aeration rate

Parameters	Aeration rate		
	1 vv^{-1} min^{-1}	2 vv^{-1} min^{-1}	3 vv^{-1} min^{-1}
Growth Parameters			
X_{max} [g L^{-1}]	2.41	3.43	3.82
μ [h^{-1}]	0.13	0.50	0.33
Production Parameter			
$P_{max(extra)}$ [U mL^{-1}]	1.48	3.79	8.94
$P_{max(cyto)}$ [U mL^{-1}]	0.66	1.95	5.75
$P_{max(peri)}$ [U mL^{-1}]	0.38	0.29	0.94
$P_{max(total)}$ [U mL^{-1}]	2.52	6.03	15.63
$Y_{p/x}$ [U g^{-1}]	1045	1758	4102
Q_p [U L^{-1} h^{-1}]	105.6	468.0	949.8

Abbreviations: X_{max}, maximal cell dry weight; m, specific growth rate; $P_{max(extra)}$, maximal extracellular phytase production;$P_{max(cyto)}$, maximal cytoplasmic phytase production;$P_{max(peri)}$, maximal periplasmicphytase production;$P_{max(total)}$, maximal total phytaseproduction;$Y_{p/x}$, U phytase produced per g biomass; Q_p, phytase production rate;$Q_{P(extra)}$, extracellular phytase production rate.

of 6.03 U mL^{-1} was obtained in the cultivation run at 2.0 v $v^{-1}min^{-1}$ after 13 hours, while cultivation at 3.0 v $v^{-1}min^{-1}$ resulted in a maximal total activity of 15.63 UmL^{-1} after 15 hours. The expression of phytase by *E. coli* was influenced by the stability of the plasmid itself, where cell division and plasmid production should be parallel in order to get higher expression rates of phytase.

Plasmid stability

Based on the data in figures (1-3), it is clearly observed that the plasmid stability in all bioreactors decreased gradually over time. As shown from Fig. 2, the plasmid that carried phytase gene was decreased slowly and finally lost of the plasmid after 12 hours of cultivation. The plasmid with the phytase gene can sustained below than

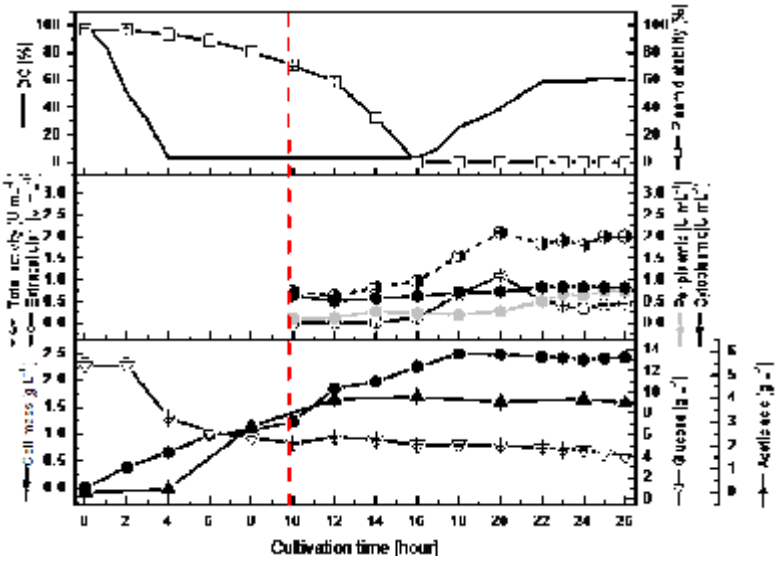

Fig. 1. Cell growth, glucose consumption, plasmid stability and distribution of phytase activity for *E. coli* BL21(DE3) cultivated at aeration rate of 1.0 $vv^{-1}min^{-1}$ with 30 mM lactose as an inducer.

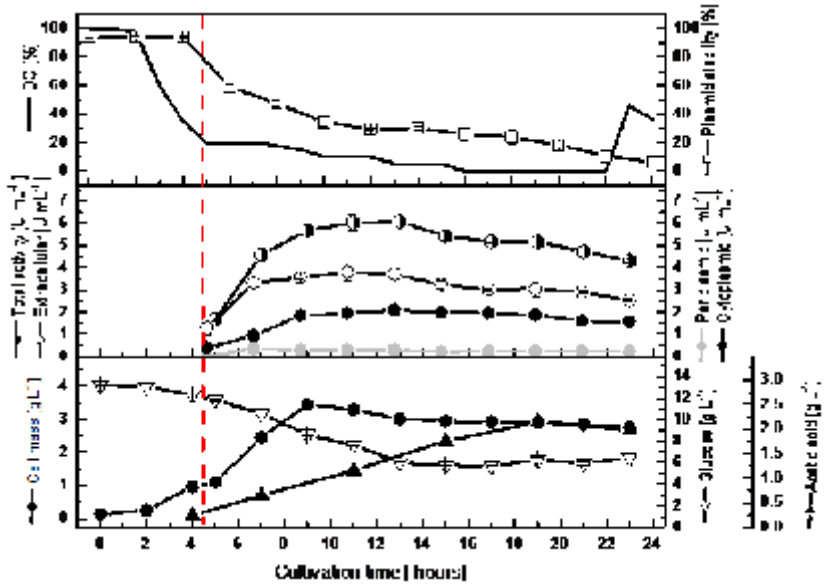

Fig. 2. Cell growth, glucose consumption, plasmid stability and distribution of phytase activity for *E. coli* BL21(DE3) cultivated at aeration rate of 2.0 $vv^{-1}min^{-1}$ with 30 mM lactose as an inducer

30% after 2 hours induction with lactose and totally lost the plasmid after 4 hours of post-induction phase. The plasmid stability for cells grown at 2.0 v $v^{-1}min^{-1}$ decreased from 93.55% at 4 hours to 3.7% at the 23 hours, while the plasmid stability for cells cultivated at 3.0 $vv^{-1}min^{-1}$ decreased from 120% at 4 hours to 32.06% at 23 hours. These results clearly show that the plasmid carrying the phytase gene in *E. coli* was more stable when the cells were cultivated at 3.0$vv^{-1}min^{-1}$. Plasmid stability is one of the most important factors affecting the yield of recombinant protein production in *E. coli* fed-batch cultivation[7]. It has been proven that plasmid stability decreased with decreasing the growth rate, this is due to the fact that the relative growth rate advantage of plasmid free-cells is higher over the plasmid containing cells under normal cultivation conditions[7]. Therefore it is expected that the plasmid stability can be increased by increasing the specific growth rate.

Table 1 summarized the most important kinetic parameters describing the growth and phytase production in stirred-tank bioreactor as affected by different aeration rates. From the data, it can be clearly concluded that aeration rate of 1 v v^{-1} min^{-1} is not suitable for phytase production using this strain. Increasing the aeration rate from 2.0 to 3.0 $vv^{-1}min^{-1}$ significantly increased the production of phytase. The phytase product increased about 136, 195 and 220% for the extracellular, cytoplasmic and periplasmic phytase, respectively. The total phytase production increased by about 134% where the concentration increased from 6.08 to 14.25 U mL^{-1} for 2 and 3 $vv^{-1}min^{-1}$, respectively. Moreover, the rate of phytase production increased by about 103%, where it

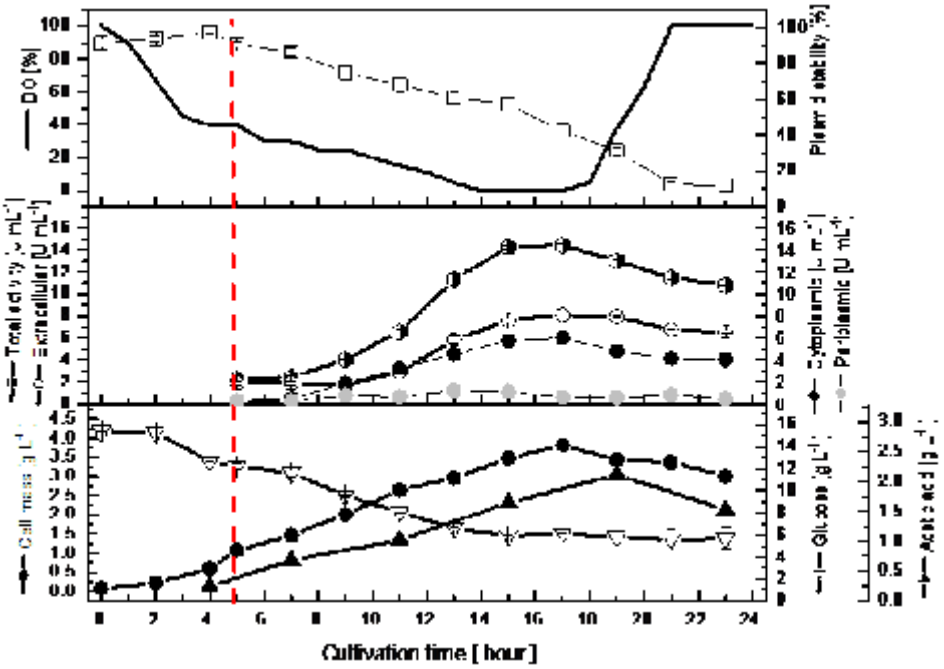

Fig. 3. Cell growth, glucose consumption, plasmid stability and distribution of phytase activity for *E. coli* BL21(DE3) cultivated at aeration rate of 3.0 $vv^{-1}min^{-1}$ with 30 mM lactose as an inducer

increased from 468.0 to 949.8 U $L^{-1}h^{-1}$ for 2 and 3 $vv^{-1}min^{-1}$, respectively. The maximal phytase yield (U phytase per g cells) increased from 2028.0 to 5088.2 upon increasing the rate of aeration from 2 to 3 $vv^{-1}min^{-1}$. This represents significant increase of the phytase production by about 151%.

CONCLUSIONS

In the present work, it has been shown that increasing the aeration rate from 2.0 to 3.0 $vv^{-1}min^{-1}$ has a remarkable effect on increasing the phytase production by recombinant *E. coli* BL21 (DE3) in stirred-tank bioreactor pilot-scale

bioreactor. The aeration did not directly affect the growth kinetics of the microbial cells, however, the increased production was due to the increased stability of plasmid, which finally resulted in a better HCDC in terms of growth and phytase production.

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