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The production of recombinant beta-galactosidase in *Escherichia coli* in yeast extract enriched medium

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SUMMARY

The productivity of *Escherichia coli* biomass and recombinant beta-galactosidase was increased in Luria broth (LB) enriched with yeast extract. In flask culture under conditions of LB limitation, yeast extract supplementation gave the highest biomass (strain HB101/pRW756) stimulation per unit of component added compared with supplementation by various amounts of amino acids, vitamins, minerals, purines/pyrimidines, tryptone, casamino acids, casein peptone or gelatin peptone. The biomass production of *E. coli* HB101/pRW756, XL-1 Blue/puc118, XL-1 Blue FF/puc118 and TB-1/p1034 cells was stimulated in fermentor-scale experiments with additional yeast extract in LB. Total beta-galactosidase production from plasmid genes in fermentor-scale experiments was increased 105.4% in XL-1 blue/puc118 cells, 365.5% in XL-1 blue FF/puc118 cells and 421.4% in TB-1/p1034 cells by 0.5%, 1% and 1% weight per volume of additional yeast extract in LB, respectively. Depending on different strains, the increase of the enzyme production was obtained either by increased biomass, or the combination of enhanced gene expression and increased biomass. Neither the biomass nor beta-galactosidase production was stimulated in N4830/p1034 cells by the increase in yeast extract concentration in the medium.

INTRODUCTION

Partial optimization of fermentation conditions for the productivity and the cost per unit of recombinant protein is becoming one of the major goals in biotechnology today, although previously attention has been paid principally to regulation of gene expression.

Luria-broth (LB, 0.5% yeast extract, 1% tryptone and 0.5% sodium chloride) is one of the most widely used media for bacterial culture and recombinant gene expression, particularly on the laboratory scale. However, since it is not the optimal me-

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dium for large-scale fermentation, improvement of the formulation may be of practical advantage. It is known from the previous work in this laboratory that glucose is one of the limiting factors for *Escherichia coli* growth in LB [9]. Supplementation of LB with glucose, to maintain a near constant glucose concentration, yielded twice the biomass obtained without glucose supplementation, because the decrease in growth rate observed at late log phase was significantly postponed [9]. In addition, the pH change in the basic direction that ordinarily begins in late log phase was controlled by the glucose addition. Here we report the results of further investigation of the limiting nutrients.

A recent study by Tsai et al. [11] demonstrated that the yield of recombinant human insulin-like growth factor (IGF-1) in *E. coli* can be increased remarkably by the feeding of high levels of organic nitrogen in the form of yeast extract plus an appropriate level of glucose in the medium during the induction period. It was proposed that the presence of high levels of organic nitrogen may result in increased product formation by protection of the peptide, IGF-1, from proteolytic degradation.

Beta-galactosidase is a tetramer of subunits of 1021 amino acid residues which is encoded by the lac operon in *E. coli*. The *E. coli* strains used in this study were beta-galactosidase defective (*lacZ* gene mutant). Recombinant beta-galactosidase or its alpha peptide were encoded by multicopy plasmids p1034 and puc118, respectively (Table 1). It was used in the present study as an example of a homologous recombinant protein made in *E. coli*.

The objective of the study was to investigate the effects of enriched medium on the expression of recombinant genes and to improve the medium for greater productivity and lower cost of recombinant proteins.

MATERIALS AND METHODS

Materials

Yeast extract and tryptone were purchased from Difco Laboratories, Detroit, U.S.A., casein peptone was obtained from Scott Laboratories, Inc., Fiskeville, Rhode Island, casamino acids and gelatin peptone were obtained from Marcor Develop-

Table 1

Bacterial strains used

Strain	Genotype
HB101/pRW756 [6]	F^-, *hsdS*20, *recA*13, *ara*-14, *pro*-A2, *lacY*1, *galK*2, *rpsL*20, *xyl*-5, *mtl*-1, *supE*44, − Amp^+
XL-1 Blue/puc118	*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, λ−, *lac*−, [F′, *proA*B, *lacI*^{q}ZΔM15, Tn*10*(tet)], [puc118, Amp^r, *lac*OPZ′]
XL-1 Blue FF/puc118	*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, λ−, *lac*−, [F′, *proA*B, *lacI*^{q}ZΔM15. Tnl*O*(tet)] [puc118, Amp^r, *lac*OPZ′]
N4830/p1034 [5]	cIts857, *lac*−, Δ *Bam*H1, [p1034, Amp^r, pL, *lacZ*]
TB-1/p1034 (derivative of JM83 [14]	*ara*, (*lac*ProAB), *strA*, r−, M^+, *thi*, ∅ 80, *lacZ* Δ M15, F−, [p1034, Amp^r, pL, *lacZ]*

ment Corp., Hackensack, NJ. All other reagents and chemicals were analytical reagent grades. A 16 liter fermentor with a 12 liter working volume, a 28 liter fermentor with a 20 liter working volume, or a two liter fermentor with a 1.2 liter working volume (all from New Brunswick Scientific) were employed for the experiments.

Strains

The bacterial strains and plasmids used in this study are given in Table 1. Initial experiments in which only the biomass concentration was determined were performed with *E. coli* HB101, a K-12 mutant transformed with plasmid pRW756 which contains a beta-lactamase gene but produces no other known recombinant protein.

Beta-galactosidase production experiments were performed with *E. coli* strain XL-1 Blue, XL-1 Blue FF, TB-1 and N4830. Strain XL-1 Blue carries a frameshift mutation in chromosomal *lacZ* gene [3]. The transforming plasmid, puc118, carries the genes for beta-lactamase, the regulatory region, and the alpha-peptide portion of the *lacZ* gene which corresponds to the first 107 N-terminal amino acids of the latter enzyme [14]. The alpha peptide has donor activity in the intracistronic complementation and leads to enzyme activity by reactivation of the alpha-acceptor *lacZ*ΔM15 gene [1] which is located on the F′ episome of XL-1 Blue [3,12]. The plasmid was introduced into XL-1 Blue cells by $CaCl_2$-mediated transformation [6].

A spontaneous mutant, XL-1 Blue FF/puc118, was isolated from XL-1 Blue/puc118 plate. Although the mutant has lower biomass yield, the plasmid DNA isolated from the mutant has the same size as puc118 DNA. Furthermore, expression of the same phenotypic properties as XL-1 Blue/puc118, i.e., ampicillin, tetracycline resistance and formation of blue colonies on indicator-plates containing IPTG and X-gal, were observed in the mutant.

Beta-galactosidase defective strain TB-1 is a derivative of strain JM83 [14], which carried a Δ(*lac-Pro*AB) chromosomal deletion and a *lacZ*ΔM15 deletion in phage $\varnothing$80 genome. TB-1 was transformed by plasmid p1034 onto which a beta-lactamase gene and *lacZ* gene had been cloned. The *lacZ* gene was placed under the transcriptional control of the lambda phage pL promoter. Beta-galactosidase was constitutively synthesized in TB-1/p1034 cells.

Strain N4830 is beta-galactosidase defective and it is a lambda lysogen [5]. The lambda repressor (product of cI_{857} gene) is sensitive to temperature (42°C). The prophage bears the ΔH1 deletion, which removes the right prophage 'att' region (the prophage-bacterial junction), therefore, prophage excision cannot occur during the thermoinduction. N4830 was transformed by plasmid p1034. Upon the thermoinduction, transcription of *lacZ* gene from the pL promoter in p1034 was derepressed.

Shaking flask culture experiments

In order to determine the limiting factor for cell growth, under conditions of glucose abundance, flask culture experiments were performed with HB-101/pRW756 and XL-1 Blue FF/puc118 in the presence of various concentrations of Luria Broth in order to determine the level at which the biomass was most sensitive to nutrient supplementation. The cell density was determined at stationary phase. A concentration that was 2% of normal LB was selected as that at which a small change of nutrient concentration could cause the greatest increase in cell density.

Therefore, the basal medium contains 0.02% tryptone (w/v), 0.01% yeast extract (w/v), 0.2% KH_2PO_4, 0.2% K_2HPO_4 (both anhydrous), 0.5% NaCl, 2% glucose and 60 mg/l ampicillin. In a series of flasks (250 ml), 50 ml of this basal medium was further supplemented with one of the following: amino acids, minerals, vitamins, purines/pyrimidines or ammonium chloride, in amounts calculated to reproduce their concentrations in full-strength LB [7]. In experiments with complex components, the basal medium was supplemented with various concentrations (0.01–0.1%) of one of the following: yeast extract, tryptone, casamino acids, casein peptone or gelatin peptone. In experiments with XL-1 Blue FF, 0.1 mM isopropyl-thio-β-D-galactoside (IPTG) was added to the medium to induce expression of beta-galactosidase.

The inoculum (1%, v/v) was an overnight culture

in LB with 60 mg/l ampicillin. The culture was allowed to grow to stationary phase with shaking at 200 rpm (amplitude = 2.5 cm) at 37°C. The turbidity (A_{650}) and pH of the culture were measured. A flask containing only the basal medium and one with full-strength LB were included as controls. Further growth experiments with various concentrations of those supplemental components that supported a significant increase in biomass were performed to determine the concentration effect. Yield and cost per unit were calculated from the initial slope of the concentration curve.

Fermentor experiments

The growth medium was full strength Luria Broth (with 60 mg/l ampicillin). In experiments with XL-1 Blue/puc118 and XL-1 Blue FF/puc118 cells, the medium also contained 0.1 mM IPTG. The enriched medium was prepared by supplementation of LB with yeast extract to a final concentration from 1.0% to 3.0%. The inoculum (1%, v/v) was an overnight culture of the appropriate strain in LB supplemented with 60 mg/l ampicillin. The pH of the medium was controlled at 7.2 by the addition of 10 N NaOH under acidic direction and either 40% phosphoric acid or 24% glucose stock feed [9] under basic direction. Temperature was controlled at 37°C for TB-1/p1034, XL-1 Blue/puc118 and XL-1 Blue FF/puc118. For N4830/p1034, the temperature was maintained at 30°C during the growth phase and was shifted to 42°C at the induction phase. Oxygen was supplied by bubbling sterilized air at 0.6 volume/min and agitation was 500, 228 and 300 rpm in the 16-l, 28-l and 2-l fermentor, respectively. The antifoaming agent was MAZU DF 60 P. The culture turbidity was measured at 650 nm, and the data were converted to dry weight by interpolation on a graph of results with similar *E. coli*. The cells in 100 ml of culture were harvested by centrifugation at 5000 rpm for 30 min, and cell dry weight was determined, after the pellet was dried to a constant weight in a vacuum freeze-dryer overnight. In the fermentation experiment for beta-galactosidase production, 10 ml of culture samples were taken from the reactor at intervals (1–2 h) for enzyme assay.

Beta-galactosidase assay

The method for enzymatic assay in whole cells was adapted from that of Schleif [10]. The cells were centrifuged at 3300 rpm for 15 min, and the pellet was resuspended in appropriate volume of assay buffer (0.1 M sodium phosphate, pH 7.0, 0.001 M $MgSO_4$, 0.1 M 2-mercaptoethanol) to give a standard turbidity (A_{650} = 1). After addition of toluene to a final concentration of 5% and *o*-nitrophenyl-galactoside (ONPG, 13.3 mM in assay buffer) to a final concentration of 2.7 mM, the mixture was allowed to react for exactly 1 min (at 30°C). The reaction was terminated by addition of 1 M sodium carbonate to a final concentration of 0.33 M and the reaction mixture was filtered with a 0.20 μm syringe filter to remove cells. Finally, the absorption of *o*-nitrophenyl was measured (A_{420}). Suitable controls for the spontaneous hydrolysis of ONPG were included. A unit of enzyme is that amount that hydrolyzes 1.0 nmol of ONPG per min.

Cost of biomass and product

The cost per unit of biomass and beta-galactosidase was the volumetric titer of the enzyme or the final biomass yields divided by the current retail price of the medium components used, fermentor use ($24–35 per run), and labor ($25 per run). Since various medium supplements were only compared in the flask culture experiments, the cost was calculated from the price of the supplement and the cell yield.

RESULTS AND DISCUSSION

Flask experiments for the limitations of E. coli growth

The biomass produced by supplementation of the basal medium with each component was compared with that in the control experiments. Additional vitamins, minerals and purines/pyrimidines produced no additional growth. Further growth experiments with various concentrations of those supplemental components that supported a significant increase in density were performed in order to determine the yield of biomass on each component and the cost of

Table 2

The effect of the supplements on HB101/pRW756 growth in flask culture (from Fig. 1)

Supplemental components in the basal medium	Yield	Cost of unit cell increase
	(A_{650}/gm)	($)
L-phenylalanine	0.017	0.12400
L-tyrosine	0.025	0.03400
L-phenylalanine + L-tyrosine	0.026	0.09300
yeast extract	0.852	0.00740
tryptone	0.244	0.01973
casamino acids	0.383	0.00925
gelatin peptone	0.212	0.02368
casein peptone	0.226	0.02366

The yield is the slope of the curves in Fig. 1. The cost per unit of biomass increase was calculated from the initial slope of the curves in Fig. 1 and the current retail price of the component (cost = price of the supplemental components used/slope of the curve).

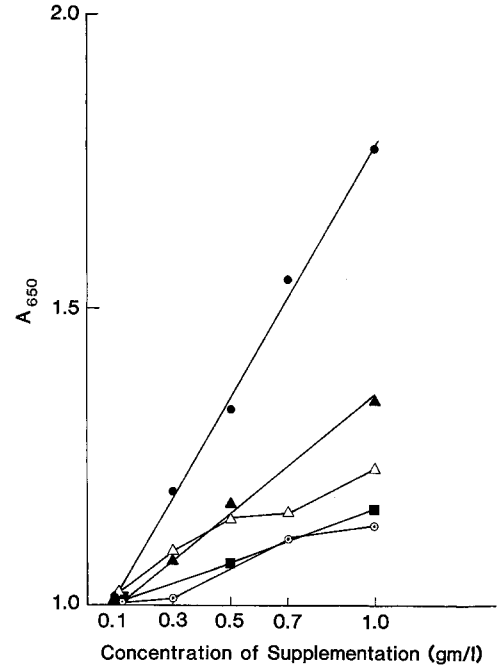

Fig. 1. The yield of HB101/pRW756 in the basal medium supplemented with various amounts of yeast extract (●), casamino acids (▲), casein peptone (△), tryptone (■), and gelatin peptone (⊙) in flask culture. The yield in the basal medium alone (A_{650} = 0.94) served as a blank.

that component per unit of biomass increase (Table 2). The results show that some of the supplemental components such as amino acids did increase the cell density but that the cost of the increase was high. The cell yields (Fig. 1, Table 2) and the cost per unit of increase in biomass (Table 2) produced by the five supplemental complex components: yeast extract, tryptone, casamino acids, casein peptone or gelatin peptone show that yeast extract supplementation resulted in the greatest yield increase, 31.5–56.4% higher than that of the other components (Fig. 1), and the lowest costs of cell production, 20–69% lower than that of the other components. It, therefore, was the most cost effective of the components studied. Based on these results, yeast extract was used as the enrichment component in scale-up experiments.

Cell production in fermentor

The results of the flask experiments were confirmed in scale-up experiments, and the maximum cost-effective concentration of yeast extract was observed at about 20 g/l (Fig. 2, Table 3). The maximum increase of biomass was 255.3%, and the maximum percentage decrease in cost was 64.1%. The decrease in yield above the maximum cost-effective concentration might result from other growth limiting factors or toxin accumulation.

Beta-galactosidase production in fermentor experiments

In fermentor experiments in which the level of beta-galactosidase was measured, the productivity of both the biomass and the total beta-galactosidase of the strains increased with increasing yeast extract, except with strain N4830/p1034. Although the biomass concentration of XL-1 blue/puc118 cells increased with supplementation by yeast extract (Table 4), the generally constant level of beta-galactosidase per unit of biomass indicates that the change of cell density resulting from the enrichment apparently has little effect on the gene expression and product activity. Nevertheless, the total productivity of beta-galactosidase was increased in this system. Production at a relatively modest level per

Table 3

The effect of yeast extract supplement on the growth of HB101/pRW756 in fermentor

Media	Final cell concentration	Net cell mass increase	Cost/ unit Biomass	% saving
	(g dry weight/l)	(%)	$/g	
LB (glc feed)	4.56	0	12.86	0
LB (glc feed) +0.5% Y.E.	5.82	27.6	10.96	14.7
LB (glc feed) +1.0% Y.E.	12.23	168.2	5.66	56.0
LB (glc feed) +1.5% Y.E.	14.74	223.2	4.74	63.1
LB (glc feed) +2.0% Y.E.	16.20	255.3	4.62	64.1

cell may be an advantageous situation in the case of some recombinant proteins, which have to be kept at relative low levels in order to minimize their possible harmful effects on cellular processes. In addition, the results with this strain may reflect the balance between the productivity of the alpha peptide and that of the remainder of the enzyme.

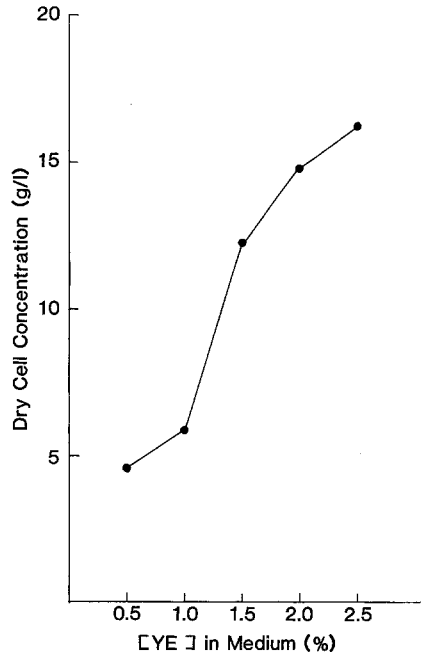

Fig. 2. Dry cell concentration (g/l) of HB-101/pRW756 in LB supplemented with various amounts of yeast extract in fermentor culture.

In the case of XL-1 blue FF/puc118 (Table 5) and TB-1/p1034 cells (Table 6), yeast extract enrichment resulted in a similar increase in biomass to that of the other two strains, although both the cell growth rate and the density at stationary phase was lower than that of HB101/pRW756 and XL-1 Blue/puc118. However, in contrast with the strains discussed above, the specific productivity of beta-galactosidase increased 172.8% (XL-1 Blue FF/puc118) and 269.2% (TB-1/p1034) with the concentration of yeast extract at 1.5% (Table 5 and Table 6, respectively). Therefore, in XL-1 Blue FF/puc118 and TB-1/p1034, the volumetric titer of beta-galactosidase was actually increased in two ways: by the increase in biomass and by the increase in the enzyme specific productivity. The percent decrease in cost per unit of activity, therefore, was higher than that of XL-1 Blue/puc118, which resulted solely from an increase in biomass.

In contrast, neither the biomass nor beta-galactosidase production in N4830/p1034 was stimulated by an increase in the yeast extract concentration in the medium (Table 7). It is not yet known whether this phenomenon is related to the fact that N4830 is a lambda lysogen. Furthermore, it is not clear whether the enhancement of beta-galactosidase specific productivity in XL-1 Blue FF/puc118 and TB-1/p1034 in yeast extract enriched medium was attributed to the cell or the plasmid or both. However, the enhancement was not observed in strain

Table 4

The effect of yeast extract supplement on the production of beta-galactosidase in XL-1 Blue/puc118 cells

Media	Yield of biomass	Total BG	BG	Increase of BG per g of biomass	Cost per unit of BG	% saving
	(gm dry weight/l)	(unit/l $\times 10^{-3}$)	(unit/g dry wt $\times 10^{-3}$)	(%)	($ $\times 10^{-3}$)	
LB (no glc added)	2	178.8	89.4		0.0308	
LB (glc feed)	5.2	397.3	76.4	0	0.0142	0
LB + 0.5% Y.E. (glc feed)	9.6	816.0	85.0	1.1	0.0084	41.17
LB + 1% Y.E. (glc feed)	8.5	725.1	85.3	1.1	0.0078	45.35
LB + 1.5% Y.E. (glc feed)	6.83	510.9	74.8	−2.1	0.0128	9.91

Table 5

The effect of yeast extract supplement on the production of beta-galactosidase in XL-blue FF/puc118 cells

Media	Yield of biomass	Total BG	BG	Increase of BG per g of biomass	Cost per unit of BG	% saving
	(gm dry weight/l)	(unit/l $\times 10^{-3}$)	(unit/g dry wt $\times 10^{-3}$)	(%)	($ $\times 10^{-3}$)	
LB (no glc added)	1.73	193.5	111.86		0.0200	
LB (glc feed)	1.70	118.3	69.61	0	0.0337	0
LB + 0.5% Y.E. (glc feed)	2.60	240.4	92.47	32.8	0.0180	46.6
LB + 1% Y.E. (glc feed)	2.90	550.7	189.90	172.8	0.0084	75.1
LB + 1.5% Y.E. (glc feed)	2.85	516.8	181.33	160.5	0.0095	71.7

Table 6 The effect of yeast extract supplement on the production of beta-galactosidase in TB-1/p1034 cells

Media	Yield of biomass	Total BG	BG	Increase of BG per g of biomass	Cost per unit of BG	% saving
	(gm dry weight/l)	(unit/l $\times 10^{-3}$)	(unit/g dry wt $\times 10^{-3}$)	(%)	($ $\times 10^{-3}$)	
LB (glc feed)	3.15	110.7	35.13	0	0.292	0
LB + 0.5% Y.E. (glc feed)	4.50	299.5	66.55	89.4	0.109	62.8
LB + 1% Y.E. (glc feed)	4.45	577.2	129.70	269.2	0.057	80.4
LB + 1.5% Y.E. (glc feed)	4.25	567.1	133.43	279.8	0.059	79.9

Table 7

The effect of yeast extract supplement on the production of beta-galactosidase in N4830/p1034 cells

Media	Yield of biomass	Total BG	BG	Increase of BG per g of biomass	Cost per unit of BG	% saving
	(gm dry weight/l)	(unit/l $\times 10^{-3}$)	(unit/g dry wt $\times 10^{-3}$)	(%)	($ $\times 10^{-3}$)	
LB (glc feed)	2.24	2754.9	1229.9	0	0.0117	0
LB + 0.5% Y.E. (glc feed)	2.52	2612.7	1036.8	−15.7	0.0125	− 6.8
LB + 1% Y.E. (glc feed)	2.35	2422.6	1030.9	−16.2	0.0136	−16.2

XL-1 Blue and N4830 into which the identical plasmids were introduced.

Preliminary evidence indicates that it is not likely that the observed results were due to the protective role of enriched organic nitrogen against proteolytic activity as in the case of IGF-1 [11]. The change of beta-galactosidase activity in XL-1 Blue FF/puc118 culture at stationary phase was measured from 0 to 60 min after the addition of chloramphenicol to the fermentor (250 mg/l) to inhibit protein synthesis [15]. The stability of beta-galactosidase in XL-1 Blue FF/puc118 in the medium supplemented with various concentrations of yeast extract in the presence of chloramphenicol was the same.

Some combination of two general mechanisms might be involved in the effect of yeast extract on the enhanced beta-galactosidase specific productivity. The enriched medium may have logistic effects in which the yeast extract provides some components necessary for the elongation of either all or certain proteins. Alternatively, some components in the yeast extract might affect certain regulatory processes for the initiation of transcription, the initiation of translation, or both.

The glucose inhibition of the inducibility of the lac operon (Epstein et al., 1975) was confirmed in flask culture experiments in which glucose concentration greater than 0.1% inhibited beta-galactosidase production. In addition, when phosphoric acid instead of glucose was used to control the pH in fermentor experiments with strain XL-1 Blue/puc118, beta-galactosidase specific activity was higher (17%) than when glucose control was used. However, the total yield of beta-galactosidase was higher with glucose supplementation (122%) because of the increase of biomass. Furthermore, the maximum glucose concentration in the media was less than 0.1% when glucose feed procedure was used, below that level shown in flask experiments not to cause repression. The explanation of the apparent repression is to be investigated.

It is shown in Tables 3, 4, 5 and 6 that the final biomass of the four strains upon yeast extract enrichment varied from 2.9 to 16.2 g dry wt/l. Furthermore, different supplemental yeast extract concentrations (0.5–1.5%) were required for minimum cost of biomass and/or beta-galactosidase in the four strains. However, the stimulative effect of the enrichment on the biomass was general (42.9–255.3% increase). Thus, the difference of the response to yeast extract concentration among them was quantitative rather than qualitative. The optimal supplemental amount of yeast extract may vary depending upon different strains and specific products to be made.

Although the mechanism of the stimulation of beta-galactosidase production remains to be determined, our results show that significant economic benefit can be obtained by the yeast extract enrichment of LB to increase both volumetric and specific productivity.

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