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Medium Optimization for Xylanase Production by Recombinant *Escherichia coli* B24

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Interest in xylanase enzyme application has led to production of xylanase from recombinant *Escherichia coli* B24, which is an economic alternative towards higher productivity. Recombinant *E. coli* used in this study is a ubiquitous bacterium containing xylanase encoding gene from *Bacillus halodurans*. We investigated xylanase production by recombinant *E. coli* using classical medium optimization. Six fermentation media had been chosen from literature for xylanase production. Afterwards, the most suitable medium was further optimized by varying the key nutrients. The final optimized cultivation medium consisted of ($g.L^{-1}$): glucose, 2.5; NH_4Cl, 0.4; KH_2PO_4, 3.0; Na_2HPO_4, 6.0; $MgSO_4.7H_2O$, 1.0. After medium optimization, maximal volumetric xylanase production (600.25 $U.mL^{-1}$) increased by about 115.22% from the initial un-optimized medium (278.9 $U.mL^{-1}$).

Keywords: Recombinant, *Escherichia coli*, Xylanase production, Medium optimization, Fermentation

Introduction

Escherichia coli is a very versatile host used in many production processes for heterologous proteins. It has many advantages; such as fast proliferation rate, ease of foreign DNA manipulation inside cells, and fast and economic production process.[1–3] Xylanase, endo-1,4-Beta-xylanase, is an industrially important enzyme, which degrades the linear polysaccharide beta-1,4-xylan into xylose[4], thus breaking down hemicellulose into xylans, which are further degraded into short-chain xylo-oligosaccharides. Xylan is one of the foremost anti-nutritional factors in common use feedstuff raw materials. Xylanases are widely used in many animal feed preparations to stimulate growth rates and improve digestibility. Xylanase thins out the gut contents and allows increased nutrient absorption and increased diffusion of pancreatic enzymes in the digesta.[5]

The production process of valuable microbial enzymes is generally optimized either through medium or process parameter optimization.[6–8] Medium optimization is traditionally carried out by adopting one-factor-at-a-time method, where each medium component is separately optimized to find out its most suitable concentration affecting production process parameters.[9] Also, this method is time-consuming, however, it is mostly favored by researchers who do not have enough facilities to carry out more sophisticated statistical optimization approaches.

In the current work, we used a recombinant *E. coli* strain harboring xylanase gene from *Bacillus halodurans*.[10] Firstly, different production media were screened to choose the most suitable one in terms of better cell growth and xylanase production. Furthermore, different medium components were investigated for the effect on production process parameters. Finally, the optimized medium composition was compared with the initial composition regarding cell growth and enzyme production kinetics.

Materials and Methods

Microorganism

E. coli B24 strain, constructed to secrete extracellular xylanolytic enzymes[11], was obtained from Dr. Rajni Hatti-Kaul from Department of

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Molecular Biophysics Centre for Chemistry and Chemical Engineering, Lund University and employed throughout this study. This recombinant *E. coli* harbors *B. halodurans* gene responsible for xylanase production.

Cell propagation

Cell culture was streaked on nutrient agar plate containing 100 µg.mL^{-1} ampicillin and was incubated overnight at 37°C for 24 hours to obtain single cell colony. A single cell colony was picked from the stock culture and inoculated in 100 mL LB medium containing 100 µg.mL^{-1} ampicillin in 250 mL-Erlenmeyer flasks. Inoculated flasks were incubated overnight at 37°C and 200 rpm. This culture was used as standard inoculums for all fermentations.[12]

Production media and cultivation conditions

Six different cultivation media were chosen based on previous literature for evaluating their effect on cell growth and xylanase production by *E. coli* B24. The composition of different cultivation media investigated is presented in Table 1. The pH of all media was adjusted to 7.0 before sterilization and carbon source was autoclaved separately before being added to the medium directly prior to inoculation. Under shake flask cultivation, all media were incubated at 37°C temperature and 200 rpm for 24 h. Samples were taken every 2 hour, and when the optical density of the culture reached 0.8, the culture was induced with 10 mM IPTG. Samples were then taken, centrifuged and kept at low temperature for further analysis.

Table 1 — Composition (g L^{-1}) of different cultivation media used for xylanase production by *E. coli* B24

Component	Medium No.					
	1	2	3	4	5	6
Tryptone	10.0	—	—	—	—	—
Yeast extract	5.0	25.0	—	—	5.0	25.0
NaCl	5.0	5.0	—	—	—	5.0
Glucose	50.0	50.0	0.02	50.0	50.0	50.0
K_2HPO_4	—	—	3.0	—	—	—
$Na_2HPO_4.2H_2O$	—	—	6.0	—	—	—
$MgSO.7H_2O$	—	—	1.0	1.0	1.0	—
NH_4Cl	—	—	1.0	—	—	—
Nutrient salt solution	—	—	—	100 mL	100 mL	—
$CaCl_2$	—	—	—	1.0	1.0	—
TES	—	—	—	1.0	1.0	—
Casein peptone	—	—	—	—	—	50.0
Reference	3	3	3	3	3	3

Optimization of different medium components

During this work, the most suitable medium composition was subjected for further medium optimization by changing the concentrations of each medium component separately. Cultivation parameters were then analyzed.

Analysis

Optical density and pH determination

Microbial cell growth was determined optically by measuring medium optical density (OD) at 600 nm[13] immediately after sampling and suitable dilutions using spectrophotometer (SPECTRONIC 200E, Thermo Fisher Scientific, MA, USA). The obtained OD was converted to dry cell mass through a liner correlation standard curve, where 1 OD_{600} is almost equivalent to 0.3 g.L^{-1}. The pH of the culture was evaluated using pH meter (Hannah pH meter HI 8424, Hanna Instrument, RI, USA).

Determination of xylanase activity

Xylanase concentration was determined using DNS methods.[14] Firstly, 2 mL of microbial sample was centrifuged for 15 minutes at 10000 rpm. The supernatant, 1 mL was pipetted into a new test tube, and then 1 mL of citric acid buffer was added. Thereafter, 1 mL of Xylan-Birchwoor was added and the mixture was immediately incubated in a water bath which at 60°C for 30 minutes. Afterwards, 2 mL of DNS reagent were added and the mixture was further incubated at 95°C for 5 minutes. Finally, tubes were cooled down in ice and the OD was measured at 540 nm. Xylanase activity was calculated by using xylose standard graph previously prepared.

Statistical analysis

Each experiment was repeated three times, and the results were presented as mean ± SD. SPSS 9.0 was used to analyze obtained data using ANOVA for comparison between different treatments, where $p \leq 0.05$ reflects statistical significance.

Results and Discussion

Screening of different cultivation media

Six different cultivation media, which were chosen based on literature (Table 1) were investigated to obtain the most suitable medium affording maximal xylanase production by *E. coli* B24. From the obtained results (Fig. 1A), it can be seen that medium 1 supports the highest xylanase production compared to other media tested. Medium 1 contained complex nutrients, which need to be avoided. From

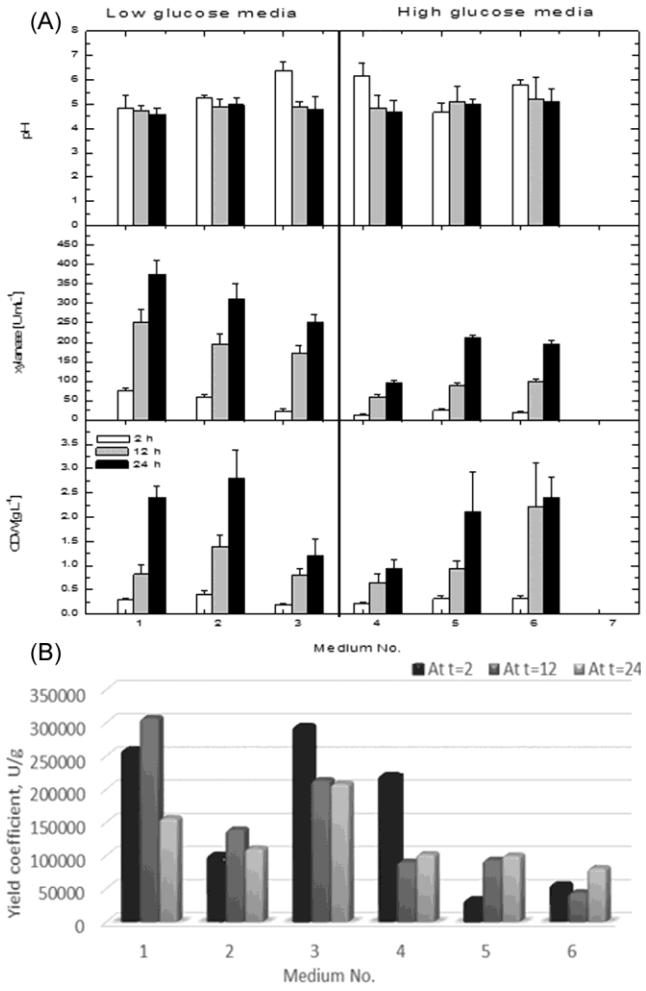

Fig. 1 — Effect of different cultivation media on xylanase production by recombinant *E. coli* after 24 hours of fermentation at 37°C and 200 rpm in shake-flasks (A: production parameters, B: yield coefficients)

yield coefficient results (Fig. 1B), it can be concluded that after 2 hours, medium 3 resulted in the highest yield coefficient. Fermentation medium 3 recorded the highest yield coefficient (295238.1 $U.g^{-1}$). Our results indicated that a combination of glucose as carbon source, NH_4Cl, KH_2PO_4, $Na_2HPO_4.2H_2O$ and $MgSO_4$ provides the highest xylanase production at the tested cultivation conditions.

It has been previously reported that monosaccharides and some disaccharides (xylose, glucose and melibiose) result in almost total inhibition of xylanase production excretion due to catabolite repression. The effect of oat spelt xylan (OSX) is proven as xylanase inducer.[15] NH_4Cl as a nitrogen source was found to be good, because it is an inexpensive source of nitrogen phosphate, and it should be kept at 1:2 ratio in order to maintain its buffering capacity. Accordingly, medium 3 was chosen for further optimization of different medium components.

Effect of different medium components on cell growth and xylanase production

During this set of experiments, different medium components in Medium 3 were tested at different concentrations to obtain the most suitable concentration enabling maximal production of xylanase enzyme. Results in Fig. 2 represent the effect of different concentrations of each component on cell growth and xylanase production. From the obtained results (Fig. 2A), it can be seen that highest xylanase production (537 $U.mL^{-1}$) was obtained after 20 h upon using 2.5 $g.L^{-1}$ of glucose. Further increase in glucose concentration significantly decreased produced enzyme. This may be related to the production of higher concentrations of lactic acid which tend to decrease cell viability and affect cell growth and consequently enzyme production[16]. On the other hand, using 2.5 $g.L^{-1}$ glucose was accompanied by complete glucose exhaustion from the cultivation medium. Also, it can be seen that pH decreased with time due to lactic acid production. After glucose exhaustion, cells started to die faster. Accordingly, further experiments were performed using 2.5 $g.L^{-1}$ glucose.

Concerning nitrogen source, NH_4Cl was tested at different concentrations (0.0-1.0 $g.L^{-1}$). Results (Fig. 2B) reveal that highest xylanase production (575 $U.mL^{-1}$) was achieved at 0.4 $g.L^{-1}$ NH_4Cl, whereas further increase in NH_4Cl concentration resulted in decreased cell growth and therefore xylanase production. The maximal CDW obtained was 1.2 g L^{-1}. Consequently, further experiments were used with 0.4 $g.L^{-1}$ NH_4Cl as the optimal nitrogen source concentration.

Finally, the effect of different concentrations of phosphate sources on xylanase production was evaluated. Since the medium contained two phosphate sources (KH_2PO_4 and $Na_2HPO_4.2H_2O$.), therefore, different combinations of both sources were tested (0.0–3.0 and 0.0–6.0 $g.L^{-1}$, respectively). Results (Fig. 2C) showed that highest xylanase production was obtained upon using 3 $g.L^{-1}$ KH_2PO_4 and 6 $g.L^{-1}$ Na_2HPO_4. These results are in good agreement with those obtained by Farliahati *et al*.[3], who obtained a highest xylanase activity upon using the same phosphate concentrations used in our work. Furthermore, highest CDW and consequently

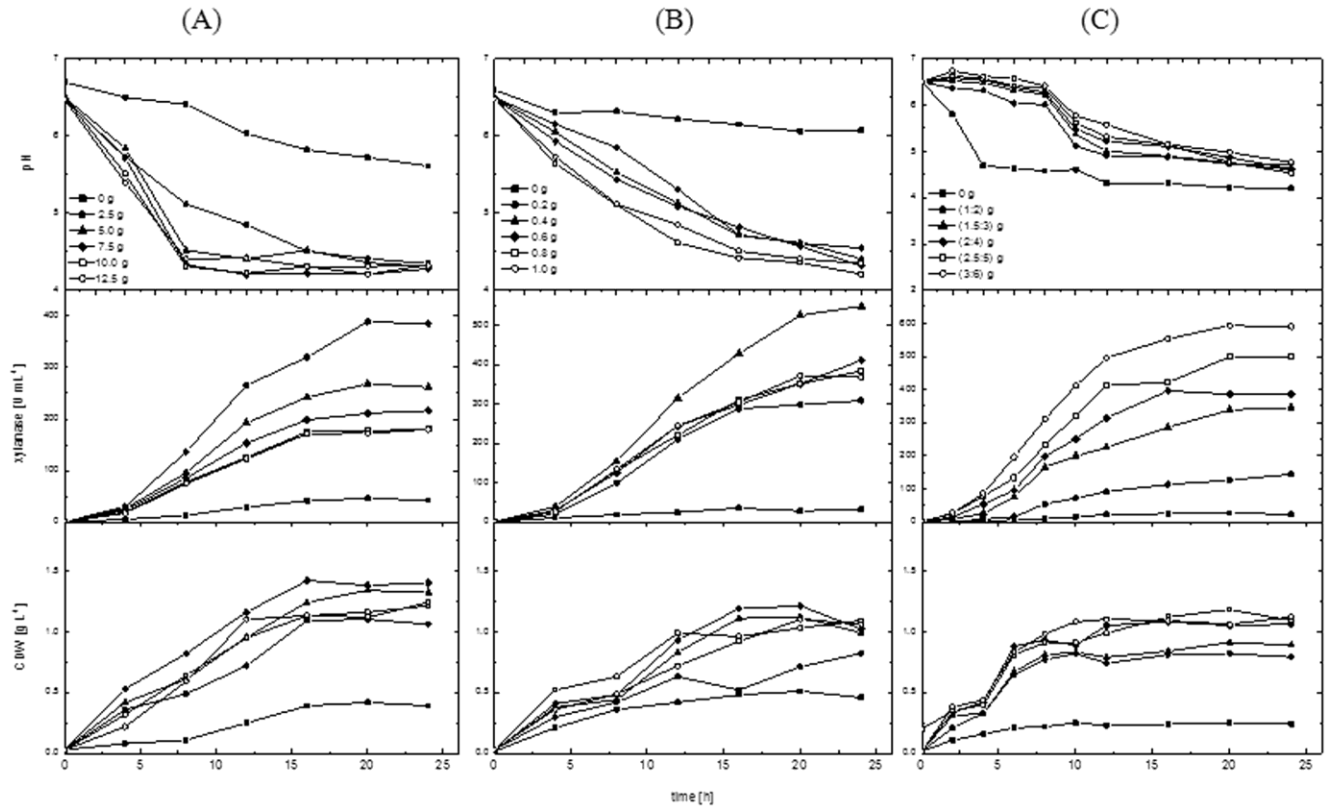

Fig. 2 — Effect of different concentrations of medium components on xylanase production by recombinant *E. coli*: (A): glucose, (B): NH_4Cl, (C): phosphate

xylanase production were obtained after 20 h of cultivation. Accordingly, the final optimized cultivation medium contained KH_2PO_4 and Na_2HPO_4 at 3.0 and 6.0 $g.L^{-1}$, respectively. Therefore, the finally optimized production medium contained ($g.L^{-1}$): glucose, 2.5; NH_4Cl, 0.4; KH_2PO_4, 3.0; Na_2HPO_4, 6.0; $MgSO_4.7H_2O$, 1.0.

Xylanase production using initial and optimized medium composition

The final part of the work was designed to follow up the kinetics of cell growth and xylanase production using both initial un-optimized and final optimized cultivation media to evaluate the effect of medium optimization on both parameters. Results presented in Fig. 3 show that cells grew exponentially in both cultivation media until reaching the stationary phase. However, the exponential phase was shorter in case of optimized medium, where cells entered the stationary phase after 10 h, while the un-optimized medium was characterized by longer exponential growth phase (up to 20 h). This can be attributed to the fact that optimized medium composition provides growing cells with their suitable nutritional requirements, and thus cells grow and consume nutrients rapidly. This was reflected on the maximal obtained cell mass concentrations in both cultivations. Un-optimized medium produced a maximal cell mass of 1.30 $g.L^{-1}$ at 20 h, after which, cell growth slightly decreased to 1.20 $g.L^{-1}$ by the end of cultivation. On the other hand, optimized medium resulted in the production of a maximal cell growth of 1.1 $g.L^{-1}$ at 10 h, which remained more or less constant by the end of cultivation (24 h). Although the maximal cell growth in optimized cultivation was about 15% lower than obtained in un-optimized cultivation, however, optimized cultivation resulted in a significant increase in xylanase production. The maximal production of 600.25 $U.mL^{-1}$ obtained at 24 h in optimized cultivation was about 2.15-folds higher than that obtained in un-optimized cultivation (278.9 $U.mL^{-1}$ at 22 h).

These results prove that medium optimization significantly improved cell growth and xylanase production parameters. Xylanase yield coefficient obtained in our current work (295238 $U.g^{-1}$) was significantly higher than that reported earlier by Farliahati *et al.*[3] (2122.5 $U.g^{-1}$). Our results are in good agreement with those previously published on

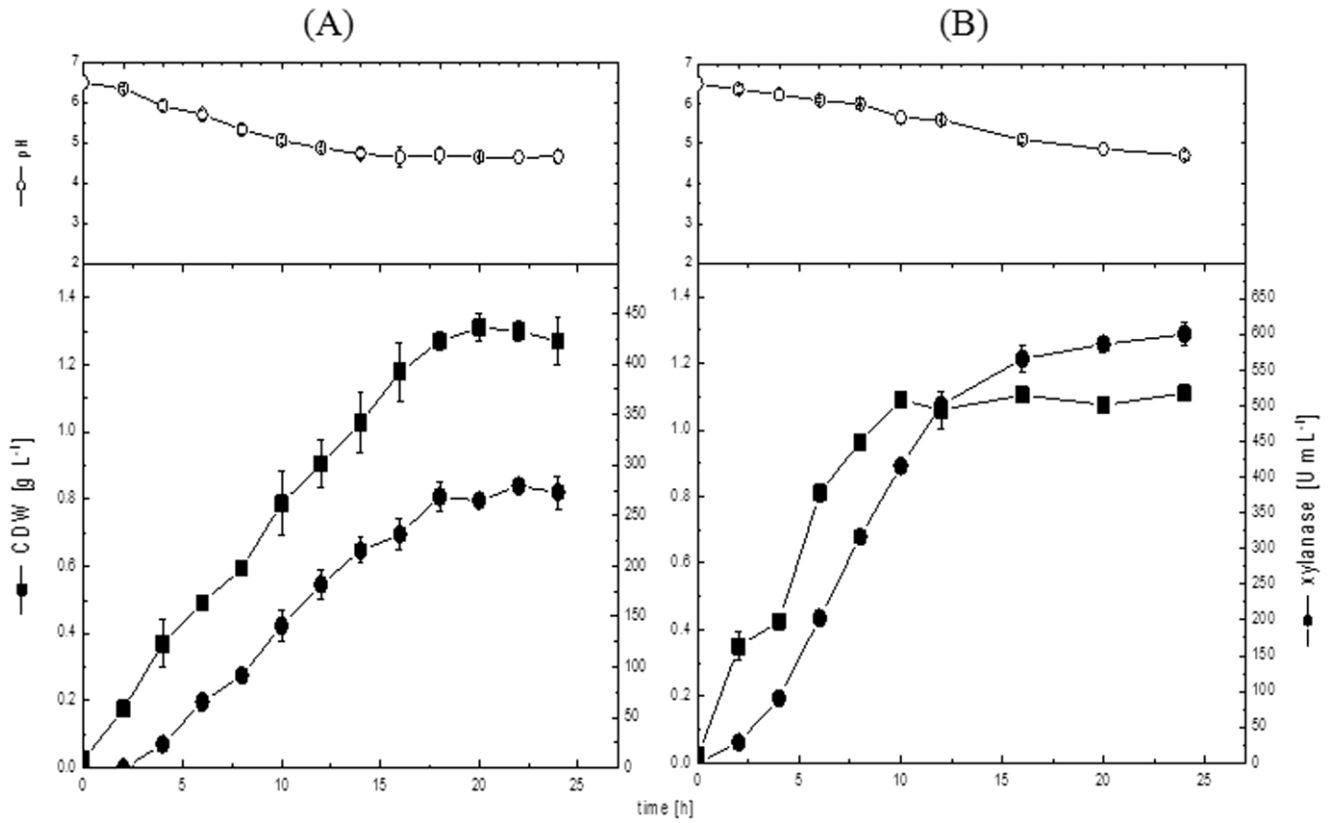

Fig. 3 — Effect of initial (A) and optimized (B) medium composition on growth kinetic and xylanase production by recombinant *E. coli*

the importance of medium optimization for increasing volumetric production of industrially valuable microbial products[8,16–20].

Conclusion

Through the current work, we were able to evaluate different cultivation media suitable for xylanase production using *E. coli* B24. The most suitable medium affording maximal xylanase production was further optimized. The final optimized medium contained ($g.L^{-1}$): glucose, 2.5; NH_4Cl, 0.4; KH_2PO_4, 3.0; Na_2HPO_4, 6.0; $MgSO_4.7H_2O$, 1.0. This medium resulted in about 2.15-fold increase in volumetric xylanase production (600.25 $U.mL^{-1}$) from the initially un-optimized medium (278.9 $U.mL^{-1}$). The current study suggests further optimization of xylanase production process through different feeding strategies as well as different cultivation parameters.

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