Effect of Uracil Addition on Proteomic Profiles and 1,3-Beta-Glucan Production in *Agrobacterium* sp.

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Abstract

With addition of uracil during fermentation, the production rate of 1,3-β-glucan(curdlan) was enhanced. Since uracil was used as precursor of UDP-Glucose, the UDP-glucose level was increased and further glucan synthesis rate was increased.

Key words: 2-DE (2 dimensional gel electrophoresis), *Agrobacterium sp.* MALDI-TOF MS. Curdlan.

Introduction

Curdlan is a biopolymer produced by *Agrobacterium* sp. ATCC31750 and insolublein water. It is composed of β-1, 3-linked glucose and synthesized under nitrogen-limiting condition [1)]. The linear structure of curdlan allows it to resist heat and other external influences, including pH. Curdlan has been used for food texture modifier and as an enhancer of flow properties of concrete[2~4)]. Curdlan was approved as a food material by the FDA (US Food and Drug Administration) in 1996.

For the improvement of curdlan productivity, several optimization strategies have been applied. For example, optimization of fermentation pH was done in other works, however the productivity still as low as 0.5g/L/hr in a batch fermenter. In the other paper, we optimized curdlan productionand the final curdlan concentration was increased up to 93g/L after 160h fermentation with addition of both sucrose (200g/L) and uracil (0.5g/L). In this report, uracil increased UDP-glucose level and it served not only as an activator of glycosyl metabolism but also as a precursor in the formation of other monosaccharides [2)].

The molecular level studies on curdlan synthesis has been publ;ished to identify curdlan synthesis enzymes. The genes essential for the production of bacterial β-1,3-glucan

have been transferred into *Agrobacterium sp.* ATCC31749[5]. Another additional curdlan related genes whose protein products occur in the cell envelope was identified by T. Karnezis(2002) and the transposon Tn*pho*Awas used as specific genetic probe[6]. Despite all these efforts, the mutants couldn't improve the productivity of the curdlan. Another method so called transcriptional profiling was introduced. It's relatively quick and easy, mRNA abundance is not always a reliable indicator of corresponding protein abundance [7-9].

In this paper, the proteomic profilechange with addition of uracill as a precursor of UDP-glucoes will be examined, We specially focused on enzyme related glucan synthesis metabolism, UTP-glucose-1-phosphate, contributed to synthesis of UDP glucose. The proteomic profilesboth in before and after uracil added condition was analyzed by 2D gel electrophoresis followed by MALDI-TOF MS.

Materials and mathods

- Strain and culture conditions

Agrobacterium sp. ATCC 31750 was used as a glucan production strain. Fermentations were carried out in a 5 L fermenter (Kobio-tech, InCheon, Korea) containing 3L of Nutrition medium (Sucrose 140g/L, NH_4Cl 4g/L, KH_2PO_4 1g/L, $MgSO_4 \cdot 7H_2O$ 0.5g/L, Trace element 10mL/L). The fermenter was inoculated with 300ml of preculture broth grown to the exponential growth phase. The initial pH was kept 7.0 with 2N HCl and 2N NaOH. The pH of fermenter was controlled to 5.5 after the cell growth and also the dissolved oxygen(DO) value was increased immediately. During the fermentation, the temperature and aeration rate were maintained at 37℃and 0.5vvm, respectively. To improve curdlan productivity, 0.5g/L Uracil was added into fermenter. Cell growth was monitored by measuring the absorbance of sampled culture at 600nm.

- Analytical methods

Cells and curdlan concentrations were determined by measuring dry weight. One ml sample from fermenter was centrifuged at 12000 rpm, for 5 min. After harvesting sample, the curdlan was dissolved with 3N NaOH solution for cell mass measurement. The sample was washed with distillated water 3 times. Thenthe pellet was dried for measuring the cell mass. After dissolving curdlan in NaOH sol-

ution, 3N HCl was added to the solution to make acid solution and then centrifuged at 8000rpm, for 15min. Supernatant was removed from tube, the remained pellet was curdlan. The curdlan was washed with distillated water 3 times and then dried for measuring curdlan concentration. The sucrose concentration was determined using the dinitrosalicylic acid method, after hydrolyzing samples with 20ul 2N HCl at 100℃ for 15min. The ammonium concentration was determined by the indophenol method.

- Sample preparation for 2-dimensional electrophoresis

Cell(2mg) were harvested by centrifugation at 4℃ at 8000rpm for 15min. Cell pellets were washed three times with Tris-HCl(40mM, pH 8.0) and then resuspended in 500ml lysis buffer(8M Urea, 4%(w/v) Chaps, 40mM Tris, 4%(v/v)protease inhibitor). After sonication for 10 sec×15times on ice, the cell debris was removed by centrifugation at 12000rpm for 60min. The protein concentration was determined by Bio-Rad protein assay kit (Hercules, CA, U.S.A.) using BSA as a standard. The supernatants were kept at -70℃ until used for 2-DE. Proteins(45ug) are resuspended in rehydration solution(8M Urea, 0.5%(v/v) Triton X-100, 0.005% Orange-G; final volume 320ul) Containing 10%(v/v) 1M DTT, 0.5%(v/v) IPG buffer(pH 3-10). Urea, Triton X-100, IPG buffer, Tris and Chaps, BSA, Orange-G were purchased from Amersham biosciences and Sigma respectively.

- Two dimensional electrophoresis and image analysis

The first step of 2-DE, isoelectric focusing, was performed on a Pharmacia Biotech IPGphor Electrophoresis System(Amersham Biosciences, Uppsala, Sweden) at 20℃. Linear pH 4-7 IPG(immobilized pH gradient) gel strips (18cm; Amersham Biosciences)were rehydratedovernight by placing the strips gel-side-down in sample-containing Rehydration solution in the IPGphor strip holder and covering with the Dry strip cover Fluid (Amersham Biosciences).

- MALDI-TOF MS (matrix-assisted laser-desorption ionizationtime-of-flight mass spectrometry) analysis and protein identification

Samples for the MAlDI-TOF MS analysis were extracted from silver-stained spots as described previously. Protein was analyzed using MALDI-TOF MS system (Voyager DE-STR, PE Biosystem, Framingham, MA, U.S.A). Spectra were calibrated

using a matrix and tryptic auto digestion ion peaks as internal standards. Peptide mass fingerprintswere analyzed using a web-based software program (MASCOT, http://www.matrixscience.com). The molecular mass and isoelectric point obtained from the protein database and from the tow-dimensional gel were then used to confirm protein identify.

- High performance liquid chromatography

For detecting the metabolite, HPLC method was used. Sample protein was extracted by sonication in 1ml lysis buffer (50mM phosphate buffer). YMC-Pack Pro C18 column(5um, 12nm, 250×4.6mm i.d.) was used within a Shimadzu LC system. The mobile phases consisted of 25mM phosphate buffer solution (18.85mM $NaH_2PO_4 \cdot 2H_2O$ and 6.15mM $Na_2HPO_4 \cdot 12H_2O$, PH 5.3). A 20ul injection of each protein sample was loaded onto the HPLC system for each analysis. The LC system was run at 1ml/min. The retention time of UDP and UDP-Glucose were 3.383min and 3.717min respectively.

Results and discussions

- Uracil addition at 84h

As shown in figure 1, ammonium was depleted after 30h and curdlan productivity was increased continuously before 72h. After 72h, the curdlan concentration was approximately 60g/L and the productivity shows no increases. At that time, Uracil(1.5g) was added and the curdlan productivity was increased and the curdlan concentration was increased up to 71.2g/L.

- 2DE analysis

The cell broth harvested at 22 hrs and pH 7.0 was used as a reference protein profiles. More than 600 spots of proteins were already identified in our previous research. In this study, we focused on the changes of key metabolic enzymes with addition of the uracil. Figure 2 shows the proteomic profiles of *Agrbacterium* sp. before and after uracil addition.

The key metabolic enzymes for curdlan synthesis identified with MALDI-TOF such as glucokinase; UTP-glucose-1- phosphate uridylytransferase; phosphoglucomutase; glucose-6-phosphate isomerase; uridylate kinase; urease gamma subunit; uracil phospharibosyltransferase. The change of protein expression level detected

with Image Master software V4.01 (Amersham Biosciences).

- *HPLC analysis*

UDP and UDP-glucose were detected by HPLC system. Before uracil addition, 24 mg/L UDP and no UDP-glucose were detected with HPLC system. After uracil addition, no UDP and 36 mg/L UDP-glucose were detected. This result indicated that uracil addition activate UTP-glucose-1-phosphate uridylytransferase catalyze the reaction from UDP to UDP-glucose.

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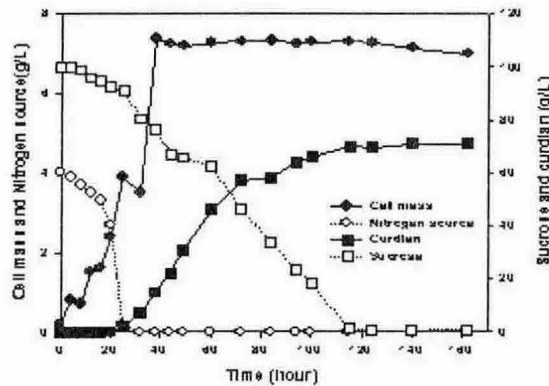

Figure 1. Effect of uracil addition(at 84 hrs) on curdlan production with *Agrobacterium* sp. ATCC 31750. pH was controlled from 7.0 to 5.5 after 25hrs fermentation.

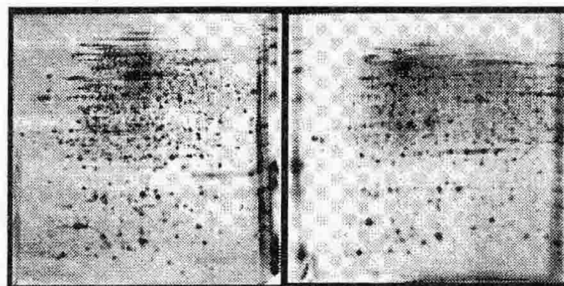

Figure 2. 2DE images (Left: before uracil addition[22h, pH7.0], Right: after uracil addition[84h, pH5.5]).

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