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High yield preparation of ganglioside GM1 using recombinant sialidase from *Cellulosimicrobium cellulans*

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ABSTRACT

Cellulosimicrobium cellulans employs extracellular sialidase to selectively convert polysialogangliosides to ganglioside GM1. We cloned this novel sialidase gene (*ccsia*) from *C. cellulans* sp. 21, and overexpressed recombinant sialidase (CcSia) protein in *E. coli* BL21 (DE3) by high cell density fermentation. The presence of an N-terminal hexa-His tag allowed for purification using nickel affinity chromatography (2.3-fold, specific activity 41.5 U/mg). As determined by gel electrophoresis and gel filtration chromatography, the molecular weight of CcSia was found to be about 75 kDa, consistent with sequence analysis (75,271 Da). CcSia transformed polysialogangliosides GD1a, GD1b and GT1b into GM1. For this reaction, the response surface approach showed that optimal conditions in a 1-L system were 2 h incubation at 32.5 °C and pH 5.2, with substrate concentrations of 10 g/L and crude enzyme concentration 1 g/L, respectively. Under above conditions, 10 g/L of ganglioside was completely converted to the product GM1 with a yield of 52%. Our studies demonstrate CcSia could be used for industrial preparation of ganglioside GM1 by the pharmaceutical industry.

1. Introduction

Recently, neurological disorders have attracted increasing attention [1,2]. However, there are limited available treatments for these medical disorders. In most instances, the use of monosialotetrahexosylganglioside (GM1) has been an effective treatment for the neurological disorders [3–6]. Therefore, large-scale preparation of GM1 using as simple a procedure as possible would be welcome by the pharmaceutical industry.

Normally, GM1 is isolated from animal brain tissue, such as pig brain, using solvent extraction followed by column chromatography [7,8]. However, the tissue content of GM1 from the total ganglioside composition is less than 20%, with the surplusage being polysialogangliosides with two or more sialic acid residues, which would be discarded during GM1 preparation process. Therefore, the yield of GM1 from this expensive protocol is relatively low. Based on structural similarities among polysialogangliosides GD1a, GD1b and GT1b, GM1 can be prepared by removal of terminal sialic acids. Although acid treatment was first introduced to convert these polysialogangliosides to GM1, this method had a low conversion rate and generated by-products like asialo GM1 (GM1 devoid of sialic acid).

Sialic acid can also be removed from polysialogangliosides by biotransformation with sialidase. Sialidases (neuraminidases, EC 3.2.1.18) cut the terminal α-2,3-, α-2,6- and α-2,8-glycosidic linkages to release sialic acid residues, are widely distributed in living organisms and participate in the metabolism of sialoglycoconjugates [9–11]. Because of high specificity and mild reaction conditions, production of GM1 using sialidase or sialidase-producing bacteria would be more promising than acid hydrolysis for large-scale preparation. To date, few reports have described the biotransformation of gangliosides using recombinant sialidase. Therefore in the present study, we cloned a ganglioside-degrading gene *ccsia* from *C. cellulans* sp. 21, produced and purified the recombinant enzyme (CcSia), and investigated its enzymatic properties. We also used response surface methodology to optimize GM1 production conditions. Overall, we have developed an approach using recombinant sialidase to effectively convert polygangliosides to GM1 in high yield.

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2. Materials and methods

2.1. Materials

A gangliosides mixture containing GT1, GD1 and GM1, used as the substrate for biotransformation, was extracted from pig brain and purified by silica gel chromatography [8]. *C. cellulans* sp. 21 was isolated from soil (Changbai Mountain, Jilin Province, China), and stored in China General Microbiological Culture Collection Center (CGMCC 7587) [12].

2.2. Analytical methods

Ganglioside biotransformation was analyzed by HPLC using a Shimadzu HPLC system (Kyoto, Japan) with an analytic Inertsil NH_2 column (DIKMA 4.6 × 250 mm, 5 μm). The eluent was: (A) acetonitrile: 5 mM phosphate buffer (pH 5.6) = 83:17 (v/v); (B) acetonitrile: 20 mM phosphate buffer (pH 5.6) = 50:50 (v/v). The gradient programs were: 0–15 min, 15% A; 15–30 min, 15–20% A; 30–34 min, 20% A; 34–50 min, 20–30% A, 50–70 min, 15% A. The flow rate was 1 mL/min and the analysis was detected at 215 nm [13]. A standard curve of GM1 was plotted in the range of 0.1–10 mg/mL. For (ESI)-MS analysis, GM1 was analyzed by API 2000 LC/MS/MS system (Applied Biosystems, Foster City, CA, USA) [14].

2.3. Cloning, expression and purification of recombinant CcSia

Genomic DNA of *C. cellulans* sp. 21 was isolated using TIANamp Bacteria DNA kit (TIANGEN, Beijing, China). The sialidase gene *ccsia* was deposited in GenBank under accession number KU521371 and amplificated by PCR method. The primers sia-F (5′-GGAATTCCATATGCACCACACGACCCTCGCCCCC-3′) and sia-R (5′-CGGGATCCTCATCAGGAGCAGTCGGTCGCCGC-3′) were designed according to the genome sequence of *C. cellulans*. The underlined nucleotides indicate restriction sites for *Nde*I and *Bam*HI, respectively. The DNA fragment obtained from the PCR was purified and inserted into pET-28a (+) vector (Novagen, Madison, WI, USA). The resulting recombinant plasmid pET28a-*ccsia* was transformed into *E. coli* BL21 (DE3) cells. Positive transformants were verified by sequencing with T7 (5′-TAATACGACTCACTATAGGGAGA-3′) and T7 terminator (5′-GCTAGTTATTGCTCAGCGG-3′) as primers.

E. coli BL21 (DE3) strain harboring recombinant pET28a-*ccsia* plasmid were grown in LB-kanamycin broth (200 mL broth in 1-L flask) at 37 °C. IPTG was added to the culture at a final concentration of 0.05, 0.1, 0.25, 0.5, or 1 mM when the $OD_{600\ nm}$ reached 0.5. The induction lasted for 20 h at 25 °C. Then the bacterial cells were harvested and disrupted by ATS-AH1500 homogenizer in lysis buffer (100 mM NaCl, 25 mM phosphate buffer, pH 7.0). The cell debris was discarded *via* centrifugation at 14,000 × *g* for 45 min at 4 °C, then the recombinant sialidase in the supernatant was purified by affinity chromatography with an Ni sepharose fast flow column (GE healthcare). Protein purity was estimated by 10% SDS-PAGE and Coomassie blue staining [15]. The native molecular mass of recombinant sialidase was estimated by gel filtration chromatography using Superose 6 10/300 GL column.

2.4. High cell density fermentation for recombinant CcSia production

High cell density fermentation was performed in a 5-L Biotech-5BG bioreactor (Baoxing Bio-Engineering, China) with a 3 L working volume. The fermentation medium included 10 g/L trypsin, 5 g/L yeast extract, 10 g/L NaCl, 12 g/L glycerol, 1.2 g/L KH_2PO_4, 1.8 g/L K_2HPO_4 and 1.2 g/L $MgSO_4$·60 mL of the *E. coli* BL21-pET28a-*ccsia* seed culture ($OD_{600\ nm}$ = 2.0) was transferred to bioreactor for recombinant enzyme production. The growth and induction temperatures during fermentation were 37 °C and 25 °C, respectively. The pH value was adjusted to 7.2 ± 0.1 using 25% ammonium hydroxide (w/w). The dissolved oxygen (DO) was maintained in a range of 20–30% air saturation. The fed medium included 280 g/L glycerol, 50 g/L trypsin and 35 g/L yeast extract. Sialidase production was initiated by the addition of IPTG (0.05 mM) to the fermentation broth when the $OD_{600\ nm}$ was 20. The induction phase lasted for 10 h. The cell density ($OD_{600\ nm}$), concentration of total protein and activity of expressed sialidase were monitored. After induction, cells were harvested, disrupted, and recombinant sialidase was purified on a Ni sepharose fastflow column (100 mL), as described in the previous section.

2.5. Enzymatic activity of sialidase

The enzymatic activity of sialidase was examined with 4-methylumbelliferyl-α-N-acetylneuraminic acid (4-MU-NeuAc) as substrate [16,17]. The 100 μL reaction mixture composed of 25 mM phosphate buffer (pH 7.0), 1 mM substrate and 2 ng recombinant sialidase, was incubated at 37 °C for 10 min. Then the reaction mixture was quenched by adding 1 M glycine-NaOH buffer (200 μL, pH 10.4), the level of fluorescence from released 4-methylumbelliferone (4-MU) was determined at 340 nm/460 nm using Infinite M200PRO microplate reader (Tecan, Männedorf, Switzerland). One unit of enzyme was defined as the amount of enzyme that generate 1 μmol of 4-MU per min.

Ganglioside hydrolysis with recombinant sialidase was carried out with 10 mg of ganglioside mixture in 1 mL phosphate buffer (25 mM, pH 7.0). Aliquots were withdrawn and assayed by Inertsil NH_2 column performed on HPLC as described above. The content of GM1 was calculated from the peak area according to a standard curve of GM1 (0.1–10 mg/mL).

2.6. Biochemical properties of recombinant sialidase CcSia

For biochemical properties assay, 4-MU-NeuAc was used as the substrate. The effects of pH and temperature on sialidase activity were determined by testing the activity at different pH values (pH 2.0–11.0) or different temperatures (20–80 °C). The pH stability of CcSia was determined by measuring the remaining activity after incubating the enzyme (2 ng) in different buffers (pH 2.0–11.0) at 37 °C for 1 h. Thermostability of CcSia was assessed by analyzing the residual activity after maintaining of sialidase solutions at various temperatures for up to 1 h.

Effect of metals and chemicals on sialidase activity was determined by pre-incubation of the enzyme in presence of metals or chemicals (final concentration 5 mM or 50 mM) at 37 °C for 1 h. Then the remaining sialidase activity was tested as described before. The sialidase activity in the absence of the compound was defined as 100%.

Kinetic studies were performed by determining the hydrolytic rate of 4-MU-NeuAc at various concentrations (10–160 μM) at 37 °C for 15 min in 25 mM phosphate buffer (pH 7.0). K_m and V_{max} values of CcSia against 4-MU-NeuAc were calculated according to Lineweaver–Burk plots by GraphPad Prism.

2.7. Optimization and scale-up production of GM1 biotransformation by recombinant sialidase

To improve ganglioside GM1 production, response surface methodology (RSM) performed on Design Expert 8.0 (Stat-Ease Inc., Minneapolis, MN, USA) was introduced to optimize the biotransformation [18,19]. The composition of pH, temperature, and substrate concentration on ganglioside biotransformation were determined using 100-mL flasks. In order to meet the need of industrial production, crude enzyme solution without purification was used for optimization and scale-up production.

A 1-L stirred tank reactor was used for scale-up production of GM1 under optimized conditions. Briefly, the 200 mL reaction mixture containing 10 mg/mL of ganglioside mixture and crude enzyme solution (final protein concentration 1 mg/mL), was reacted at 32.5 °C and

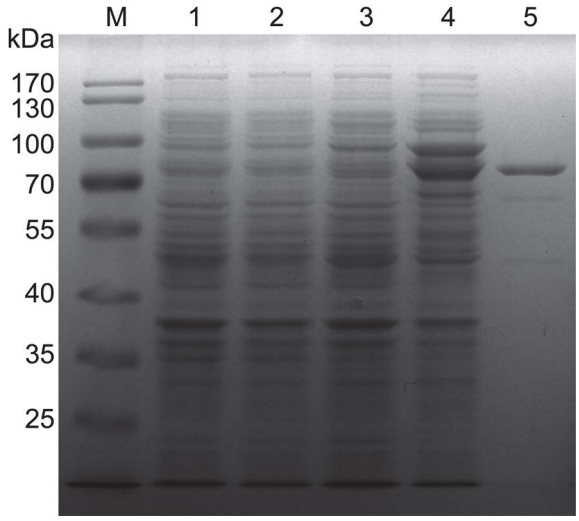


Fig. 1. SDS-PAGE analysis of CcSia on 10% separating gel. Lane 1, culture lysate of *E. coli* BL21-pET28a before IPTG induction; lane 2, culture lysate of *E. coli* BL21-pET28a after induction; lane 3, culture lysate of *E. coli* BL21-pET28a-*ccsia* before induction; lane 4, culture lysate of *E. coli* BL21-pET28a-*ccisa* after induction; lane 5, recombinant sialidase purified from Ni sepharose fastflow column; M, PageRuler Prestained Protein Ladder (Thermo Scientific).

100 rpm in 50 mM Na_2HPO_4-citrate buffer (pH 5.2). After 2 h reaction, sample was held at 90 °C for 5 min to remove the inactivated protein. The supernatant was dialyzed and freeze-dried, and used as the transformed material. The transformed material was dissolved in chloroform–methanol (4/1, v/v), and loaded onto silica gel chromatography. The column was washed by the solvent of chloroform–methanol (4/1, v/v) to remove impurities, and finally eluted by chloroform–-methanol (5/4, v/v) to give purified product. GM1 content in transformed material and purified product was analyzed by using HPLC as described before.

3. Results and discussion

It had been reported that ganglioside GM1 can be prepared from polysialogangliosides by biotransformation with various sialidase-producing bacteria. Previously, the marine bacterium *Pseudomonas* sp. strain YF-2 was used as the microbial biocatalyst for preparation of GM1. In a typical experiment, 500 mg of the crude ganglioside fraction was converted after 3 days of transformation [20]. In another study, soil bacterium *B. casei* YZ-1 was used in a 50-L microbial transformation system with crude pig brain gangliosides as substrate [21,22]. Many other sialidase-producing microorganisms have been reported, such as *Arthrobacter ureafaciens*, *Clostridium perfringens*, and *Vibrio cholera* [23–25]. Because these microorganisms are pathogenic, they are generally undesirable for production of GM1. Therefore, we sought to use a non-pathogenic microorganism to produce sialidases. In our previous study, we used bacterium *C. cellulans* sp. 21 to transform polysialogangliosides into GM1 [12]. However, we felt that the biotransformation process would be more efficient by using recombinant sialidase, primarily because of the ease of enzyme production, use of stable transformation conditions, and simplified product purification.

3.1. Gene cloning and analysis of C. cellulans sp. 21 sialidase gene

Here, we cloned a ganglioside-hydrolyzing sialidase gene from the genomic DNA of *C. cellulans* sp. 21. The sialidase gene consisting of 2217 nucleotides encodes a polypeptide of 738 amino acid residues. Multiple alignment of CcSia with the homologous sialidase from the NCBI database is presented in Fig. S1. The sequence of *ccsia* showed identity with putative sialidase sequences available in the NCBI database, such as *Kribbella catacumbae* (WP_020389291.1) (76%), *Micromonospora viridifaciens* (1EUR_A) (75%), *Ornithinimicrobium pekingense* (WP_022921610.1) (61%), *Leifsonia aquatica* (WP_021757764) (51%), *Corynebacterium glutamicum* (WP_011897290) (43%), and *Actinomyces europaeus* (WP_016444821) (42%). Previously, it was reported that the sequence Ser-X-Asp-X-Gly-X-Thr-Trp was highly conserved in bacterial sialidase sequences [26]. Similarly, the conserved sequence in CcSia was present as Ser-Asp-Asp-His-Gly-Ala-Thr-Trp.

3.2. Expression of recombinant sialidase in the flask and bioreactor

The gene *ccsia* was subcloned into pET-28a (+), then the *C. cellulans* sp. 21 sialidase was expressed in *E. coli* BL21 (DE3) cells. Different IPTG concentrations were tested in the range of 0.05–1 mM for protein expression. Results showed that the IPTG concentrations tested did not significantly affect cell growth or protein expression (Fig. S2). CcSia was easily produced by induction with 0.05 mM IPTG at 25 °C. Under above induction conditions, we got a total protein concentration of 0.58 g/L and a specific activity of 8.0×10^3 U/L. SDS-PAGE analysis showed that bacteria harboring recombinant plasmid pET28a-*ccsia* produced a high yield of soluble sialidase, which was not detectable in cultures of bacteria harboring the empty pET-28a vector (Fig. 1). Recombinant sialidase was purified by affinity chromatography. Typically, about 21 mg of pure His_6-tag fusion CcSia was obtained from 200 mL of LB culture in a 1-L flask. After one-step affinity chromatography, the specific activity of CcSia was 38.5 U/mg (Table 1).

To improve production of the recombinant sialidase, high cell density fermentation was introduced using a 5-L bioreactor. The $OD_{600\ nm}$ reached 57 after 10-h induction. For the high cell density fermentation, we got a total protein concentration of 8.3 g/L culture, and a specific activity of 1.48×10^5 U/L culture. This was much higher than that produced using the 1-L flask. Approximately 5.3 g purified recombinant sialidase was obtained from a 3.5-L culture, and this highly-efficient expression of recombinant CcSia makes its industrial application possible for gangliosides biotransformation.

Table 1
Expression and purification of recombinant sialidase from *E. coli* BL21-pET28a-*ccsia*.

	Volume (l)	Protein (g)[a]	Total activity (kU)[b]	Specific activity (U/mg)	Yield (%)	Purification fold
1-l flask						
Crude culture lysate	0.2	0.1165	1.6	13.7	100	1.0
Ni sepharose fastflow	0.015	0.021	0.8	38.5	50.5	2.8
5-l bioreactor						
Crude culture lysate	3.5	28.9	519	18.0	100	1.0
Ni sepharose fastflow	0.26	5.3	220	41.5	42.4	2.3

[a] Protein was quantified according to the Bradford method using bovine serum albumin (BSA) as standard (Bradford 1976).
[b] Enzymatic activity was determined with 4-MU-NeuAc as a substrate.

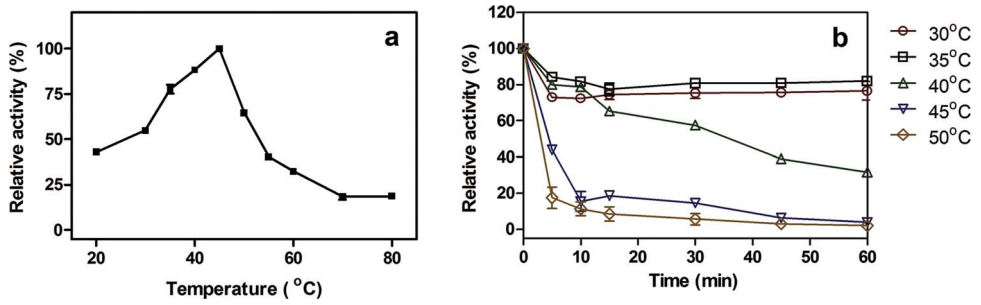


Fig. 2. Effect of temperature on activity (a) and stability (b) of recombinant sialidase using 4-MU-NeuAc as substrate. The optimal temperature (■) was determined at different temperatures from 20 to 80 °C. The maximum activity obtained was defined as 100%. The thermo-dependence of recombinant sialidase was assayed by incubating the enzyme for 1 h at different temperatures. The activity of the enzyme before incubation was defined as 100%, results shown are the mean ± SD of three independent assays.

3.3. Biochemical characterization of CcSia

The molecular weight (Mw) of CcSia was 75 kDa as determined by SDS-PAGE (Fig. 1), in agreement with that deduced from the sequence (75,271 Da). This indicated that recombinant sialidase CcSia is a moderate ganglioside-degrading enzyme, which is less than the Mw of sialidases from *Pseudomonas* sp. strain YF-2 (110 kDa) [20], and NanH from *P. multocida* (80 kDa) [27], but larger than the Mw of NA1 (65 kDa) and NA2 (54 kDa) from *A. nicotianae* [28], neuraminidase from *M. viridifaciens* (41 kDa) [29], and NeuA3 from *Streptomyces avermitilis* (38 kDa) [30]. The native molecular weight of recombinant CcSia was calculated to be approximately 80 kDa by gel filtration (Fig. S3), which indicated that CcSia is a monomeric protein.

Recombinant CcSia was most active at 45 °C (Fig. 2a), with the activity above 60 °C being less than 30% of that at 45 °C. Thermal stability analysis demonstrated that greater than 80% of its activity remained after incubating for 1 h at temperatures below 35 °C. When the temperature was higher than 45 °C, stability decreased dramatically (Fig. 2b). After incubated at 45 °C for 10 min 80% of its activity was lost. Long-term storage testing demonstrated CcSia exhibited remarkable stability at 4 °C, with over 90% activity remaining even after storage for 30 days. Using 4-MU-NeuAc as substrate, enzymatic activity of purified CcSia was investigated ranging from pH 2.0 to 11.0 where it is relatively stable. Even though CcSia showed the highest enzyme activity from pH 4 to pH 6, it was maximal at pH 5 (Fig. 3). Enzyme activity was observed to be greater than 60% after 1 h incubation.

Enzyme activity can be influenced by some metals and non-ionic surfactants. The effect of different compounds on sialidase activity was investigated. As shown in Table 2, the enzymatic activity of CcSia was strongly inhibited by Fe^{3+} and SDS at a concentration of 5 mM, whereas other metal ions and chemicals did not significantly affect its activity. At a concentration of 50 mM, nearly all metals and chemicals tested inhibited sialidase activity of CcSia, especially Ba^{2+}, Fe^{3+}, Cu^{2+}, Hg^{2+} and SDS.

Initial rate were determined using 4-MU-NeuAc as substrate at different concentrations and analyzed by using a Lineweaver–Burk plot. Apparent K_m and V_{max} were found to be 20 μM and 75 μmol/min/mg, respectively (Table S1). The apparent turnover rate (k_{cat}) and k_{cat}/K_m values were 94 s^{-1} and 4685 mM^{-1} s^{-1}, respectively.

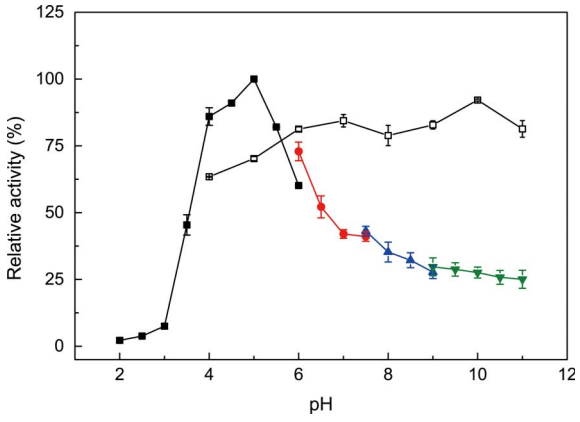


Fig. 3. Effect of pH on activity (solid symbols) and stability (hollow symbols) of the recombinant sialidase. The optimal pH was determined using 4-MU-NeuAc as a substrate at different pH values. Buffers used were as follows: pH 2.0–6.0, sodium acetate buffer (■); pH 6.0–7.5, Na_2HPO_4–NaH_2PO_4 buffer (●); pH 7.5–9.0, Tris–HCl buffer (▲); pH 9.0–11.0 Gly-NaOH buffer (▼). The maximum activity obtained was defined as 100%. The pH stability (□) of the enzyme was determined by incubating the enzyme for 1 h at 37 °C in different buffers within the pH range 4.0–11.0. The activity of the enzyme before incubation was defined as 100%. Results are presented as means ± SD (n = 3).

3.4. Conversion of the ganglioside mixture to GM1 by using recombinant sialidase

Hydrolysis using a natural ganglioside mixture was performed to determine the hydrolytic activity of CcSia. Ten milligrams of pig brain ganglioside mixture was incubated with recombinant sialidase. HPLC (Fig. 4) and TLC (Fig. S4) analysis showed that after 2 h incubation, the gangliosides GD1a, GD1b and GT1b in the mixture were converted to GM1, which suggested that the NeuAcα2, 3-linkage bound to terminal galactose in GD1a/GT1b, and the NeuAcα2, 8-linkage bound to NeuAc in GD1b/GT1b were digested by CcSia (Table 3). Asialo GM1 was not detected, even after a prolonged cultivation period, which suggested that the NeuAcα2, 3-linkage directly bound to internal galactose in gangliosides was not digested. Therefore, sialic acids were cut from the polysialogangliosides, not from GM1. These results verified that CcSia is a true ganglioside-hydrolyzing enzyme with high selectivity for GM1 production. The transformed product was further identified by ESI-MS. The characteristic negative ions $(M-H)^-$ were found at m/z 1544.85 and m/z 1573.6, which correspond to GM1 with different ceramides (Fig. 5).

Table 2
The effects of metal ions and reagents on the activity of recombinant CcSia.

Metal ions or reagents[a]	Relative activity (%)[b]	
	5 mM	50 mM
K^+	104.54 ± 1.94	47.30 ± 0.20
Ca^{2+}	79.63 ± 1.66	42.31 ± 1.01
Na^+	90.69 ± 1.31	56.12 ± 2.43
Mg^{2+}	99.57 ± 1.87	35.20 ± 2.22
Fe^{3+}	45.67 ± 0.55	0.02 ± 0.00
Cu^{2+}	88.91 ± 4.02	6.82 ± 1.93
Hg^{2+}	104.64 ± 0.22	8.82 ± 0.47
Ba^{2+}	110.34 ± 4.16	20.91 ± 2.22
Mn^{2+}	123.03 ± 3.19	52.01 ± 3.52
DTT	103.52 ± 3.90	75.23 ± 2.49
EDTA	77.59 ± 4.46	54.05 ± 3.32
SDS	1.77 ± 0.75	1.85 ± 0.55

[a] The metal ions were used in the form of chlorides.
[b] The relative activity was expressed with respect to the control without metal ions or reagents.

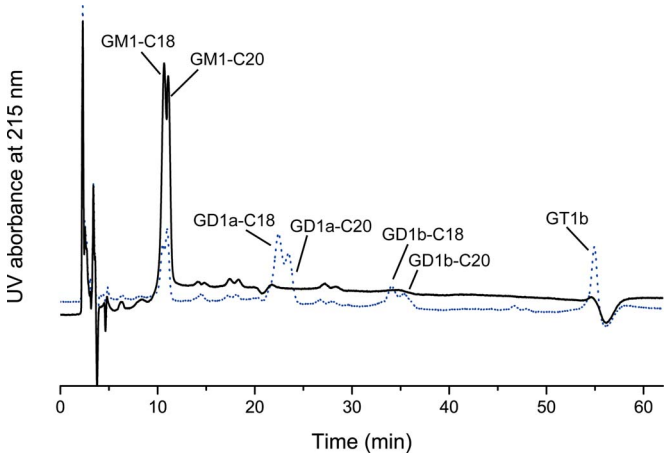


Fig. 4. HPLC analysis of the transformed product from gangliosides mixture by recombinant sialidase. Blue dot represents the gangliosides substrate; black solid line represents the transformed product. HPLC analysis was performed using a NH_2 column as described in Section 2.

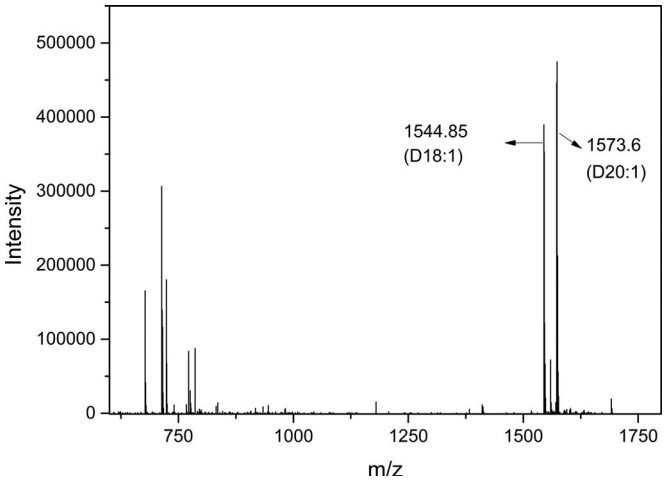


Fig. 5. The ESI-MS result of transformed product from gangliosides mixture. The sample was dissolved in MeOH and detected in the negative-ion mode.

3.5. Biotransformation optimization with RSM and scale-up production of GM1

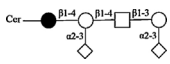
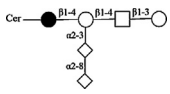
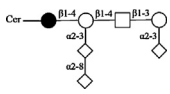
RSM was introduced to determine the optimal conditions for maximum GM1 production. First, main effects and interactions of different factors on GM1 production were investigated (Table 4). The second-order polynomial model for GM1 yield is shown in Eq. (1):

$$Y = 46.53 + 6.58A - 1.87B - 2.88C + 0.33AB + 3.35AC - 0.21BC - 7.07A^2 - 1.49B^2 + 1.16C^2 \quad (1)$$

where Y is the GM1 yield (%), and A, B, and C represents the pH, temperature (°C), and substrate concentration (g/L). The model terms were regarded as significant when the values of "Prob > F" less than 0.05. In this case, A, B, C, AC, A^2, B^2 were significant. The model F-value of 31.58 for GM1 yield was statistically significant at Prob < 0.0001. The R^2 value (coefficient of determination) was calculated to be 0.976, indicated that only 2.4% of the variability could not be explained by this model.

The relation between the three factors and GM1 production was demonstrated by the response surface plot (Fig. S5). The optimal conditions were calculated according to the canonical analysis to be: temperature 32.5 °C, pH 5.22, and substrate concentration of 10 g/L. To operate the biotransformation conveniently, the optimal pH was adjusted to 5.2. Under the conditions, the highest GM1 yield predicted from the RSM model was 51.4%, and the verified GM1 yield was 52.1% (Table 3), very close to the yield predicted by the model. The scale-up production of GM1 was carried out using the ganglioside mixture as substrate. Following biotransformation for 2 h, 2 g of ganglioside mixture yielded 1.8 g of transformed product, in which the content of GM1 was 52%. After purification using silica gel chromatography, approximately 920 ± 15 mg of GM1 was obtained, with a purity > 90%.

Table 3
Biotransformation efficiency of gangliosides by recombinant sialidase CcSia.

Ganglioside	Structure[a]	Before transformation (%)[b]	After transformation (%)
GD1a		31.9 ± 0.3	ND[c]
GD1b		7.4 ± 0.3	ND
GT1b		8.8 ± 0.6	ND
GM1		13.5 ± 0.3	52.1 ± 0.4

[a] ●: Glucose; ○: Galactose; □: N-acetylgalacosamine; ◇: N-acetylneuraminic acid; Cer: ceramide.
[b] The transformation efficiency was detected by HPLC method with NH_2 column as described in Section 2.
[c] ND: not detected.

4. Conclusions

Overall, a novel ganglioside-degradation gene *ccsia* was cloned from *C. cellulans* sp. 21 and successfully expressed in *E. coli*. A peak sialidase production of 846 U/mL culture was obtained in a 5.0-L fermenter by high cell density fermentation. Purified sialidase transformed polysialogangliosides GD1a, GD1b and GT1b to ganglioside GM1. The optimum conditions for a 2 h transformation in a 1-L system were found to be a temperature of 32.5 °C, pH 5.2, and substrate and enzyme

Table 4
Effect of three variables on GM1 production.

Run	pH	Temperature (°C)	Substrate concentration (mg/mL)	GM1 yield (%)
1	5.0	35.0	20.0	45.5
2	6.0	40.0	20.0	41.9
3	4.0	30.0	20.0	34.7
4	5.0	30.0	30.0	45.0
5	5.0	40.0	10.0	47.8
6	6.0	35.0	10.0	47.5
7	4.0	40.0	20.0	29.3
8	5.0	40.0	30.0	41.9
9	5.0	35.0	20.0	47.6
10	5.0	35.0	20.0	48.8
11	6.0	35.0	30.0	48.1
12	5.0	30.0	10.0	50.1
13	6.0	30.0	20.0	46.0
14	5.0	35.0	20.0	46.6
15	4.0	35.0	10.0	39.8
16	5.0	35.0	20.0	44.3
17	4.0	35.0	30.0	27.1

concentrations of 10 g/L and 1 g/L (crude enzyme), respectively. Using our procedure, we found that 10 g/L ganglioside mixture was completely converted to GM1 with a yield of 52%. These results make it potentially useful for large-scale ganglioside GM1 production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2017.04.010.

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