J. Appl. Glycosci., **65**, 51–56 (2018)
doi: 10.5458/jag.jag.JAG-2018_0005

Regular Paper

Cellulase Production of *Trichoderma reesei* (*Hypocrea jecorina*) by Continuously Fed Cultivation Using Sucrose as Primary Carbon Source

(Received August 2, 2018; Accepted September 29, 2018)
(J-STAGE Advance Published Date: October 19, 2018)

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Abstract: To expand the range of soluble carbon sources for our enzyme production system, we investigated the properties of sucrose utilization and its effect on cellulase production by *Trichoderma reesei* M2-1. We performed batch cultivation of *T. reesei* M2-1 on sucrose and related sugars along with cellobiose, which was used as a cellulase inducer. The results clearly revealed that the hydrolysis products of sucrose, *i.e.* glucose and fructose, but not sucrose, can be used as a carbon source for enzyme production. In a 10-day continuous feeding experiment using invertase-treated sucrose/cellobiose, the fungal strain produced cellulases with a filter paper-degrading activity of 20.3 U/mL and production efficiency of 254 U/g-carbon sources. These values were comparable with those of glucose/cellobiose feeding (21.2 U/mL and 265 U/g-carbon sources, respectively). Furthermore, the comparison of the specific activities clearly indicated that the compositions of both produced enzymes were similar. Therefore, enzymatically hydrolyzed sucrose can be utilized as an alternative carbon source to glucose in our enzyme production system with *T. reesei* M2-1.

Key words: *Trichoderma reesei* (*Hypocrea jecorina*), cellulase production, soluble carbon source, sucrose, invertase

INTRODUCTION

Lignocellulosic biomass is the most abundant renewable resource on earth. Its bioconversion into fuels or chemicals is considered to be among the most effective strategies for solving environmental problems caused by the use of fossil fuels without competition with food/feed production.[1)2)] Enzymatic hydrolysis of lignocellulosic biomass into fermentable sugars, such as glucose and xylose, is a key step for the production of biofuels and other chemicals. However, the high cost of hydrolytic enzymes remains the major hurdle to the implementation of economically friendly biorefineries. *On-site* production of enzymes is considered an appropriate strategy for reducing enzyme costs due to avoidance of expenses incurred for transportation, preservation, and storage of the product.[3)] Enormous research efforts have been focused on the reduction of the *on-site* production cost of enzymes through isolation and improvements in various strains of microorganisms and increasing the productivity and efficiency of enzymes using inexpensive media, *etc*.

The filamentous fungus, *Trichoderma reesei*, an anamorph of *Hypocrea jecorina*, is a well-known producer of hydrolytic enzymes for lignocelluloses, *i.e.,* cellulases and hemicellulases. Numerous investigations of the enzymatic properties of hydrolytic enzymes and gene expression mechanisms of this fungus have been conducted to utilize the enzymes it produces for saccharification of lignocellulosic materials. Furthermore, multiple cellulase-overproducing mutants of this fungus, some of which have already been industrially used, have been obtained worldwide. Insoluble substrates such as crystalline cellulose and pretreated lignocellulosic materials are frequently used as carbon sources and enzyme inducers for production of cellulase/hemicellulase by *T. reesei* strains. However, this fungus cannot directly assimilate these materials into their cells. Thus, the production of cellulases/hemicellulases has been considered to be induced by soluble sugars derived from these insoluble materials. Cellobiose, which is the primary product generated by cellulose degradation by cellulases, exhibits cellulase-inducing activity. However, glucose, the product of the degradation of cellobiose by β-glucosidases, strongly inhibits cellulase production.[4)] Furthermore, soluble monosaccharides released from hemicellulose, D-xylose, or L-arabinose, induce hemicellulolytic enzymes. Nevertheless, D-xylose was reported to act as both an inducer and repressor of several xylanase genes depending on its concentration.[5)] Cellulase/hemicellulase induction in *T. reesei* strain can be strongly influenced by the levels of these sugars, which can constantly vary depending on several cultural factors, such as enzyme composition and activities and the status of insoluble lignocellulosic materials. Thus, it is difficult to control the quality and quantity of the enzymes produced by *T. reesei* strains using insoluble ma-

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terials as carbon sources and inducers.

We previously reported the development of an efficient system for production of cellulases and hemicellulases by continuous feeding cultivation of a *T. reesei* mutant that is able to produce cellulases in the presence of glucose using soluble sugars such as glucose, xylose, and cellobiose.[6)7)] The aim of these previous experiments was the elaboration of a quality/quantity-controlled enzyme production system. In this system, both maintenance of high cellulase productivity and control of the composition of cellulolytic/hemicellulolytic enzymes were ensured by management of the feeding parameters of the soluble sugars used. This can be advantageous for the cost-effective production of tailored enzymes in a diversity of bioconversion processes. On the other hand, several reports claimed that the enzyme production was still expensive in cases in which the ratios of the carbon sources utilized in the cultivation were relatively high.[8)9)] For example, in a techno-economic analysis, Humbird *et al.*[8)] suggested that more than 50 % of the expenses incurred for enzymatic production of bioethanol can be attributed to the use of glucose as a carbon source. Therefore, the utilization of inexpensive carbon sources can significantly contribute to the cost-effectiveness of enzymatic production.

Sucrose is a common and convenient-to-use carbon substrate that is often generated by the sugar-processing industry as sucrose-rich byproducts, *i.e.,* sugarcane molasses.[10)] Thus, sucrose and sucrose-rich byproducts can be considered candidates for cost-effective carbon sources for enzymatic production. In the present study, we investigated the properties of sucrose utilization and its effect on cellulase production in *T. reesei* M2-1 to broaden the range of carbon sources for our enzymatic production system using continuously fed cultivation with soluble sugars. Moreover, we developed an efficient production system of *T. reesei* cellulases using sucrose as a main carbon source.

MATERIALS AND METHODS

Fungal strain and materials. *T. reesei* ATCC66589, purchased from American Type Culture Collection (ATCC; Manassas, VA, USA), and the mutated strain M2-1, a UV-irradiated mutant of *T. reesei* ATCC 66589 that can produce cellulases in media with glucose as the sole carbon source,[6)] were used. Carboxymethyl cellulose (CMC), birchwood xylan, *p*-nitrophenyl β-D-lactopyranoside (*p*NPL), *p*-nitrophenyl β-D-glucopyranoside (*p*NPG), *p*-nitrophenyl β-D-xylopyranoside (*p*NPX), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Invertase (from Yeast, 4 Wako invertase U/mL) was supplied from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Potato dextrose agar medium was manufactured by Difco Laboratories Inc. (Franklin Lakes, NJ, USA).

Cultivation of the strains. Basal medium (BM)[11)] was used for cultivation of *T. reesei* after the supplementation with carbon source(s). The enzyme production of *T. reesei* was studied at a final working volume of 1.2 L in a 2-L jar fermentor (MDL N-2L, B. E. Marubishi Co., Ltd., Tokyo, Japan). For seed cultivation, conidia of each strain (10^7 conidia) were inoculated into 100 mL of BM containing 20 mg/mL of glucose in a 500-mL flask, and then incubated by rotation (180 rpm) at 28 °C for 3 days.

The seed culture was inoculated into 1.1 L of fresh BM containing 15 mg/mL ammonium sulfate and carbon sources (i) sucrose/cellobiose: 24-g sucrose and 2.4-g cellobiose and (ii) glucose/fructose/cellobiose: 12-g glucose, 12-g fructose, and 2.4-g cellobiose. The medium was inoculated with the seed culture, followed by incubation at 28 °C at 300 rpm at 1 vvm (volumes of air per volume of liquid per min) of aeration for 3 days. During cultivation, pH was maintained at 4.0–4.2 using HCl and $NH_4(OH)$. Aliquots of the culture (10 mL) were collected at appropriate intervals, and the supernatant was recovered by centrifugation (6,000 × G for 15 min at 4 °C) to determine the sugar and protein concentrations and enzymatic activities.

For the cellulase preparation by continuous feeding, *T. reesei* M2-1 was cultivated in a 5-L jar fermentor (BNR-5L, B. E. Marubishi). Seed culture of the fungal strain (400 mL; 100 mL × 4 flasks) was inoculated into 2.5 L of fresh medium containing 20 mg/mL of glucose, followed by cultivation at 28 °C, 500 rpm, and 1 vvm of aeration. After 1-day cultivation, throughout the 10-day enzyme production period, a sugar solution was continuously added to the culture at a rate of 1.74 g-sugar/h. Two types of sugar solution were used in this experiment (i) glucose/cellobiose: glucose (100 mg/mL) and cellobiose (20 mg/mL), and (ii) invertase-treated sucrose/cellobiose: sucrose (100 mg/mL) and cellobiose (20 mg/mL) containing invertase (10 U/L) that had been incubated at 25 °C for 1 day prior to its use as a feeding solution. The culture medium was maintained at pH 4.0 for the first 3 days of cultivation and at pH 5.0 thereafter. An aliquot of the culture (350 mL) was collected each day, and clarified by centrifugation (4,000 × G for 20 min at 4 °C). Next, a portion of the supernatant was analyzed to determine the sugar and protein concentrations and enzymatic activities.

Assays. The combined activity of Cel7A and Cel7B (Cel7s), which are two primary components of the *T. reesei* cellulase system, and the activities of β-glucosidase (BGL) and β-xylosidase, were determined as *p*NPL-, *p*NPG-, and *p*NPX-hydrolyzing activities, respectively, by monitoring the release of *p*-nitrophenol (*p*NP) from the substrates at 405 nm. Reaction mixtures containing 1 mM substrate and the enzyme in 50 mM sodium acetate buffer pH 5.0 were incubated at 50 °C for appropriate durations. Then, the reactions were terminated by the addition of the same volume of 1 M sodium carbonate. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of *p*NP from the substrate per min. Endoglucanase (EG) and xylanase activities were measured on carboxymethyl cellulose (1 % w/v) and birchwood xylan (0.5 % w/v), respectively. The reaction was performed in 50 mM sodium acetate buffer with pH 5.0 at 50 °C. The reducing sugars obtained in the enzymatic reaction were quantified by the Somogyi–Nelson method.[12)13)] One unit of EG and xylanase activities was defined as the amount of the enzyme that produced 1 μmol of reducing sugars per minute in glucose and xylose equivalents, correspondingly. Additionally, the

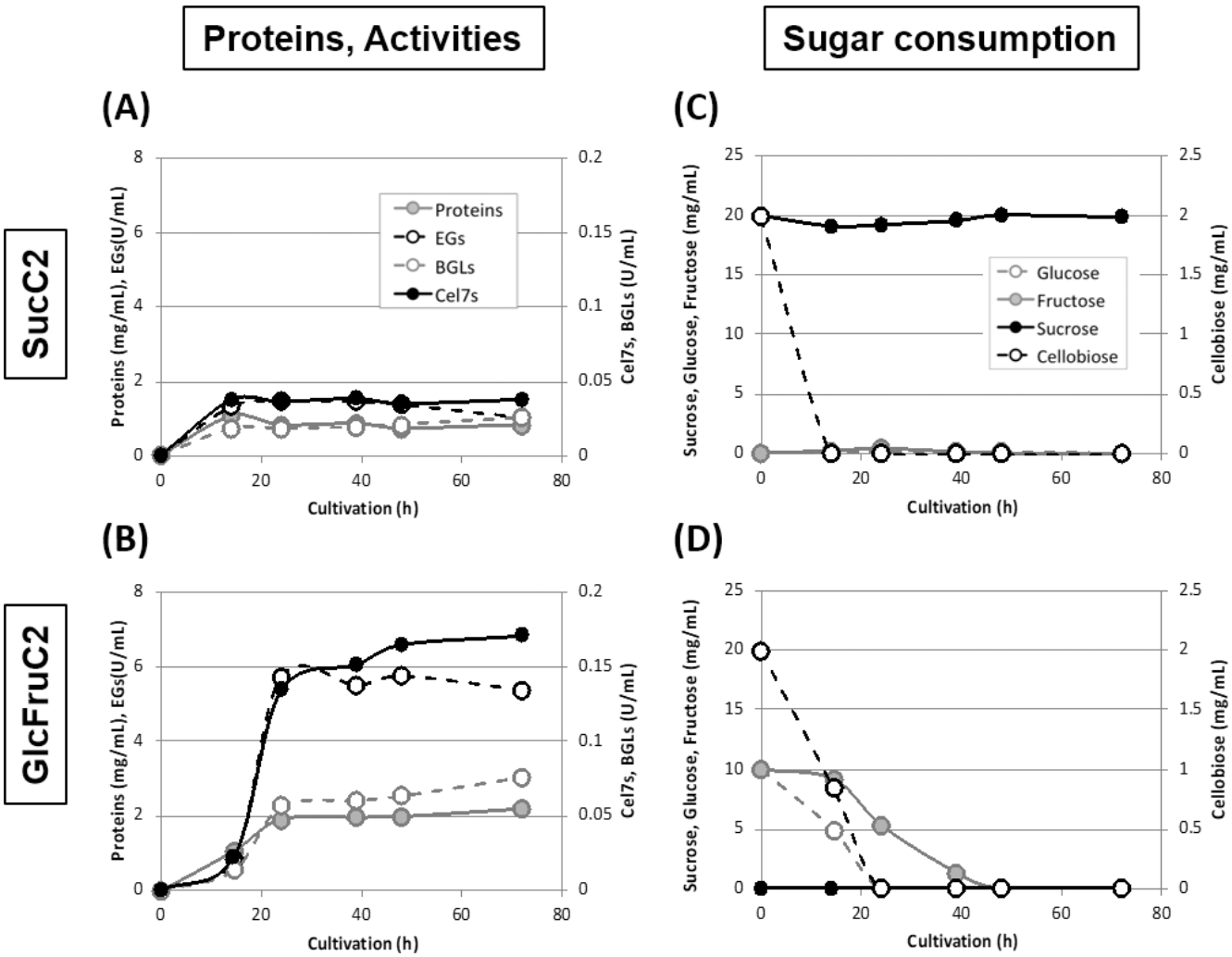

Fig. 1. Enzyme production and sugar consumption profiles during *T. reesei* M2-1 cultivation on SucC2 and GFC2.
T. reesei M2-1 was cultivated on sucrose and cellobiose (SucC2; 20 and 2 mg/mL, respectively), or glucose, fructose, and cellobiose (GlcFruC2; 10, 10, and 2 mg/mL, respectively) for 72 h. The detailed characteristics of the conditions are described in Materials and Methods. Protein concentrations and enzymatic activities on (A) SucC2 and (B) GlcFruC2. Sugar consumption on (C) SucC2 and (D) GlcFruC2.

filter paper-degrading activity (FPA) was measured according to the IUPAC recommendations.[14] Protein quantification was performed with a DC Protein Assay Kit (Bio-Rad Laboratories, Inc., Richmond, CA, USA) based on the method reported by Lowry[15] using BSA as a standard.

The sugar concentrations in the culture were analyzed using a high-performance liquid chromatography system (Prominence UFLC, Shimadzu Corporation, Kyoto, Japan) equipped with a refractive index detector (RID-10A, Shimadzu). Samples were desalted and separated at 60 °C with distilled water used as a mobile phase (0.6 mL/min) by a Deashing Micro-Guard pre-column and an Aminex HPX-87P column (Bio-Rad), respectively. For the measurements of sucrose and cellobiose concentrations, the culture was treated with β-glucosidase (E-BGOSAG; Megazyme International Ireland, Wicklow, Ireland) prior to the HPLC analysis to hydrolyze residual cellobiose because the peaks of sucrose and cellobiose could not be completely separated under these conditions. The peak area of the treated samples was regarded as the concentration of sucrose, and the cellobiose concentration was estimated from the subtraction of the peak areas between untreated and treated samples.

RESULTS AND DISCUSSION

Weak cellulolytic activities in the medium were detected in the *T. reesei* M2-1 strain cultivated on sucrose/cellobiose (Fig. 1A). In this treatment, the concentration of sucrose in the culture medium did not change during the cultivation, whereas that of cellobiose rapidly decreased, indicating that the strain cannot utilize sucrose as a carbon source (Fig. 1C). On the other hand, the cellulolytic enzymes were produced by the strain M2-1 cultivated on the sugar mixture of glucose, fructose and cellobiose (Fig. 1B). In this cultivation variant, all sugars were completely consumed within 48 h, whereas the strain seemed to preferentially utilize glucose and cellobiose over fructose (Fig. 1D). These results clearly indicate that the hydrolysis products of sucrose, *i.e.,* glucose and fructose, can be used as a carbon source for enzyme production by *T. reesei* M2-1. Reportedly, *T. reesei* QM6a, which is an ancestral strain of many mutants, including M2-1, does not utilize sucrose as the sole carbon source,[16][17][18] which is supported by the lack of invertases in *T. reesei*.[17][19] Druzhinina *et al*. investigated the growth of several *T. reesei* strains, including some wild-type and mu-

Table 1. Production efficiency and specific activities of *T. reesei* M2-1 using continuous feeding cultivation.

	Suc(In)C2	GlcC2
FPase (FPU/mL)	20.3	21.2
Production efficiency (FPU/g carbon)	254	265
Specific Activity (U/mg protein)		
FPase	0.46	0.47
Endoglucanase (EG)	3.5	3.4
β-Glucosidase (BGL)	0.25	0.24
Xylanase	6.8	6.2
β-Xylosidase	0.032	0.027

tant strains such as QM9414, on 95 carbon sources.[20] In this report, all fungal strains exhibited exceedingly poor growth on sucrose, whereas that on fructose, glucose, and cellobiose was faster. *T. reesei* M2-1 suitably utilized glucose, fructose, and cellobiose but did not utilize sucrose, which is in agreement with the case of the several wild-type strains used in the study conducted by Druzhinina *et al.*.[20] Furthermore, it is reported that fructose, as well as glucose, does not promote cellulase formation in *T. reesei* strains, QM9414 and ATCC66589.[21][22] The strain M2-1 grown on the sugar mixture of glucose, fructose and cellobiose produced 2.2 mg/mL of proteins with 0.17 U/mL of Cel7s activities (Fig. 1B), and these values were comparable to those on the sugar mixture of glucose and cellobiose (2.6 mg/mL of proteins with 0.18 U/mL of Cel7s activities). These results indicate that fructose works for cellulase production as effectively as glucose.

Next, we performed cellulase production by the continuous feeding strategy using sucrose and cellobiose as a carbon source and a cellulase inducer, respectively. The results of the batch cultivation revealed that the strain M2-1 utilized glucose and fructose, which are the degradation products of sucrose by invertase, and produced cellulases (Fig. 1). Thus, the sugar solution containing sucrose and cellobiose was treated by invertase prior to the feeding (invertase-treated sucrose/cellobiose: Suc(In)C2). Following invertase treatment, the sucrose in the sugar solution was completely converted into glucose and fructose (data not shown). As shown in Fig. 2A, the proteins and cellulolytic activities in the continuously fed culture of M2-1 linearly increased after the 3-day cultivation, indicating stable cellulase production. After 10-day production, the strain finally produced cellulases in the treatment with 20.3 U/mL of FPA. The production efficiency of FPA at 10-day cultivation was 254 U/g-carbon sources (Table 1). Similar patterns of the production of proteins and activities were obtained in the mixture of glucose and cellobiose (GlcC2) used as a feeding solution (Fig. 2B). After 10 days of GlcC2 feeding, the concentration and production efficiency of FPA were 21.2 U/mL and 265 U/g-carbon sources, respectively (Table 1). These values were comparable with those established in the treatment with Suc(In)C2 feeding. Furthermore, the specific activities of the cellulolytic and hemicellulolytic enzymes in the final cultures in the Suc(In)C2 feeding and GlcC2 feeding were almost identical (Table 1), indicating that the composition of the produced enzymes were extremely similar. These results clearly indicate that fructose, which coexists with glucose and cellobiose, rarely affects the amount and composition of the produced enzymes in the system. Both glucose and fructose were established to be rapidly utilized as carbon sources in some strains of *T. reesei*[20] and our mutant strain also consumed both sugars. From the perspective of sugar utilization properties and enzyme composition produced, fructose is a good carbon source for enzyme production using the continuously fed system. However, the strain preferentially utilized glucose over fructose when both sugars coexisted in the culture (Fig. 1). As shown in Fig. 2, fructose was detected in the continuously fed culture of Sun(In)C2, in which fructose accumulated in the culture in the early phase, whereas neither glucose nor cellobiose were detected. This accumulation could be attributed to the imbalance in feeding and consumption of the sugars. The density of mycelia increased during the early phase under these cultivation conditions, with continuous feeding of sugars at the same rate after 1-day cultivation. It is noteworthy that in the case of GlcC2-feeding, all added sugars were utilized shortly after feeding because of the rapid consumption of glucose and cellobiose by the fungus. Conversely, in the Suc(In)C2 feeding, because of slower utilization of fructose compared with glucose, it is presumed that the added amount of fructose may surpass the sugar consumed by the fungus until the mycelia were sufficiently grown. After the mycelia were fully grown (assumed to be approximately 3 days),

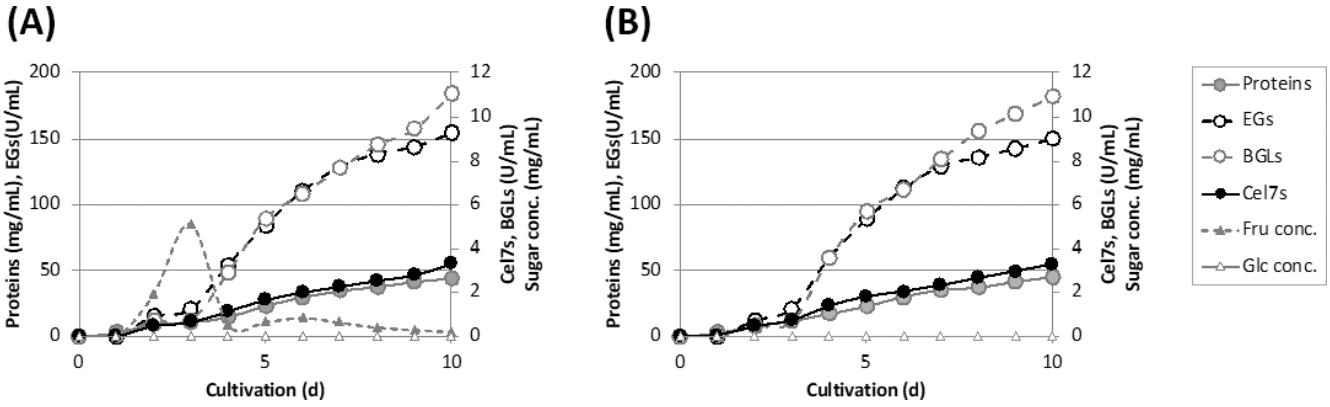

Fig. 2. Enzyme production profile from *T. reesei* M2-1 by continuous feeding of Suc(In)C2 (A) and Glc/C2 (B). The detailed feeding strategies are described in Materials and Methods.

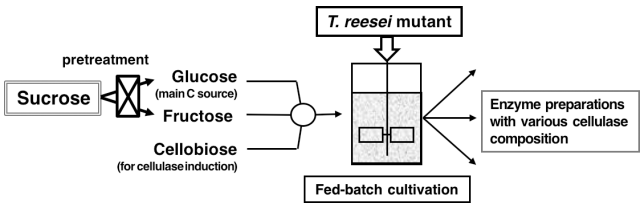

Fig. 3. Schematic diagram of the enzyme production system discussed in this paper.

the capacity of sugar consumption would exceed the fed amount. Because excessive accumulation of fructose may lead to a decline in enzyme productivity, further rigorous control of the sugar-feed conditions in the case of Suc(In)C2-feeding, such as the appropriate timing to start sugar supply and feeding rate of the sugars, is required.

In this study, we investigated the availability of sucrose in our *T. reesei* M2-1 enzyme production system and found that enzymatically hydrolyzed sucrose was utilized as an alternative carbon source of glucose (Fig. 3). Sucrose and its hydrolysate are obtained from the food- and sugar-processing industry as sucrose-rich byproducts such as molasses. Recently, Ellilä *et al.* reported the development of a system for cellulase production by *T. reesei* using sugarcane molasses and soybean hulls.[23] In this investigation, the *T. reesei* VTT-BR-C0020 strain produced sufficient enzymes on soybean hulls and hydrochloric acid-treated molasses. The authors also engineered *T. reesei* to express an invertase gene from *Aspergillus niger*, which permitted the fungus to utilize sucrose directly from sugarcane molasses provided as a carbon source. The results in this report strongly suggest that the molasses can be a valuable sucrose-rich carbon source for our continuous feeding enzyme production system. Sucrose and its hydrolyzed sugars also could be acquired from strained lees of palm and sweet sorghum, wastes of syrups, fruits from the food industry, *etc.* The use of an appropriate combination of sucrose-rich materials, based on the specific regional/local industry characteristics, as a soluble carbon source in our continuously fed cultivation system, can lead to a decrease in the cost of enzyme production. For the application of these materials into our system, we are going to carry out the investigation of the inhibitory effects on the enzyme productivities as well as the optimization of feeding condition of these sugars. Furthermore, in our previous study,[7] we used hemicellulase inducers such as xylose and arabinose along with glucose and cellobiose in the enzyme production by a *T. reesei* mutant cultivated in a continuously fed cultivation system. Importantly, in this examination, we could control the activity levels of hemicellulases in the cellulase preparations produced with high production efficiency of cellulases. The hydrolysate of lignocellulosic materials contains cellobiose and xylose/xylooligomer, which induce cellulase and hemicellulase production by *T. reesei*, respectively, along with the use of glucose as a primary monomeric sugar. Several reports on *T. reesei* cellulase production through the use of hydrolysates of lignocellulosic materials have been previously published.[24][25][26] The hydrolysates of lignocellulosic materials combined with sucrose-rich sources such as soluble sugars in a continuously fed system for cultivation of *T. reesei* M2-1 could enable cellulase production from low-cost materials. To further develop our enzyme production system using soluble sugars, we are currently conducting further studies, including investigations on the effect of other soluble sugars on the enzyme productivity of *T. reesei* mutants and applied research of the usefulness of sugar-containing industrial wastes and byproducts. Additionally, we are improving strains that are suitable for our enzyme production system using several raw soluble materials.

ACKNOWLEDGMENTS

We would like to express our gratitude to Ms. M. Tabuse, Ms. K. Yamanaka, and Ms. X.F. Lv for their excellent technical assistance. This work was supported by grants provided by the SIP-Lignin Project, Technologies for creating next-generation agriculture, forestry and fisheries under the Cross-ministerial Strategic Innovation Promotion Program (SIP) administered by Council for Science Technology and Innovation (CSTI), Japan.

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