

Concurrently Produce a Novel High-Potency Sweetener (Glycyrrhetic Acid 3-O-Mono- β -D-glucuronide) and Lignocellulolytic Enzymes using Plant Entophytic *Chaetomium Globosum* DX-THS3 by Solid-State Fermentation

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Keywords: Glycyrrhetic acid 3-O-mono- β -D-glucuronide, *Chaetomium globosum* DX-THS3, solid-state fermentation, lignocelluloses-degrading enzymes

Posted Date: June 2nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-563893/v1>

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Abstract

Licorice straw was used for the first time as a medium for glycyrrhetic acid 3-O-mono- β -D-glucuronide (GAMG) and lignocellulosic enzyme production via solid-state fermentation (SSF) of endophytic fungus *Chaetomium globosum* DX-THS3. Under optimal fermentation conditions, the percent conversion of glycyrrhizin reached 90% in 15 days, whereas the control needed 35 days to achieve the same result. The productivity of optimization ($P=2.1 \text{ mg}\cdot\text{g}^{-1}\cdot\text{day}^{-1}$) was 2.33-fold that of non-optimization ($P=0.9 \text{ mg}\cdot\text{g}^{-1}\cdot\text{day}^{-1}$). Meanwhile, high activities of filter paper enzyme (FPase) (234.6 U/g), carboxymethyl cellulase (CMCase) (29.25 U/g), xylanase (72.52 U/g), and β -glucuronidase activity (264.17 U/g) were obtained faster than those in the control during SSF. Our study provides a novel and efficient strategy for GAMG production and indicates *C. globosum* DX-THS3 as a potential producer of lignocellulolytic enzymes.

1. Introduction

Glycyrrhizin (GL) is a major bioactive component of industrial crop *Glycyrrhiza* (Pandey & Ayangla, 2017) with numerous valuable physiological properties, including antioxidative (Michaelis et al., 2011), antiviral (Ito et al., 1988), anticancer (Huang et al., 2016), and anti-inflammatory (Wang et al., 2015a) action. GL can efficiently inhibit the coronavirus disease (COVID-19) virus, and it is commonly considered a potential chemical for the cure of COVID-19 (Baillly & Gérard Vergoten, 2020; Murck, 2020). However, GL has low absorption and strong adverse effects in humans and animals (Akao, 2000; Wang et al., 2013). GL can be transformed into glycyrrhetic acid 3-O-mono- β -D-glucuronide (GAMG), which possesses improved biological activities and safety and higher solubility, by hydrolysis of one of the terminal glucuronic acids of GL (Lin et al., 2009; Li et al., 2017). Furthermore, GAMG is about 5 and 1000 times sweeter than GL and sucrose (Mizutani et al., 1994), respectively, and tastes better (less aftertaste) than GL. Therefore, GAMG has valuable application, especially in the food industry. However, efficient approaches for large-scale GAMG production are unavailable thus far.

GAMG can be produced by chemical synthesis and biotransformation. However, chemical approaches generally present several disadvantages, including the requirement of strong and harsh conditions, ineffective costs, poor selectivity, and environmental pollution (Brieskorn & Lang, 1978). Compared with chemical approaches, the biosynthesis of GAMG significantly has more potential and advantages because of its excellent substrate selectivity, high yields, mild reaction conditions, and eco-friendly status. The efficient biosynthesis of GAMG involves the use of β -glucuronidase (GUS) (Zou et al., 2013; Xu et al., 2018; Wang et al., 2013; Park et al., 2005). At present, GUSs are mainly screened from microorganisms (Zou et al., 2013; Xu et al., 2018; Wang et al., 2013; Park et al., 2005). However, most of reported GUSs exhibit a low hydrolytic selectivity and cause further transformation of GAMG to GA. On the other hand, GUS is generally prepared by microorganic fermentation. The isolation of GUS for biosynthesis of GAMG has several challenges, such as complicated processes and harsh cultivation conditions. Therefore, an efficient, simple, and feasible approach for the production of GAMG must be developed.

In general, most enzymes are produced by microorganic submerged fermentation (SF). GUS can also be produced by SF of *Aspergillus terreus* Li-20 (Xu et al., 2018), *Streptococcus* LJ-22 (Park et al., 2005), and *Penicillium purpurogenum* Li-3 (Zou et al., 2013). However, their applications in the manufacturing industry have been met with several processing challenges, including low productivity, especially in terms of ineffective costs of the mass production of enzymes. Compared with SF, solid-state fermentation (SSF) can offer several advantages, including low equipment requirements, simple culture processing, and the use of widely available and inexpensive lignocellulosic residues (usually crop straws and wastes) as substrates (Pandey, 2003). Therefore, SSF is very suitable to produce highly bioactive products, such as enzymes. Thus, the utilization of SSF for large-scale production of GAMG should be considered. However, to date, the use of SSF to produce GAMG has not been investigated.

Endophytic fungi are an ecological group of fungi that is present in living plant tissues without initiating any external symptoms. Endophytic fungi have attracted research attention because they can secrete a number of secondary metabolites, including bioactive chemicals (Suryanarayanan et al., 2012). In addition, endophytic fungi can be utilized as plant material, for example, crop straw, for use as a medium for SSF to produce valuable products, such as bioactive chemicals and enzymes (Prajapati et al., 2018; Natália et al., 2018; Deswal et al., 2011). In our previous study, an endophytic fungus *Chaetomium globosum* DX-THS3 was isolated from Dongxiang wild rice (*Oryza rufipogon* Griff.) (Wang et al., 2015b). Furthermore, a GUS with specificity and highly transformable GL to generate GAMG was screened from *C. globosum* DX-THS3 (Zhang et al., 2020), and genome analysis showed abundant genes coding lignocellulose-degrading enzymes harbored in the *C. globosum* DX-THS3 genome (unpublished). Therefore, in this study, *C. globosum* DX-THS3 was utilized with licorice straw as a medium for SSF. We aimed to i.) investigate the feasibility of GAMG production by *C. globosum* DX-THS3 via SSF and provide an efficient strategy for large-scale production of GAMG; ii.) analyze the lignocellulose-degrading enzymes, such as carboxymethyl cellulase (CMCase), FPase, xylanase, and GUS, during SSF to simultaneously obtain high-activity enzymes and demonstrate *C. globosum* DX-THS3 as a potential candidate for producing lignocellulose-degrading enzymes.

2. Material And Methods

2.1 Chemicals and strains

Licorice straw was purchased from Inner Mongolia, China. Standard GL and GA samples (purity $\geq 98\%$) were purchased from Sigma Chemical Co. (USA). Standard GAMG (purity $> 98\%$) was prepared and identified via carbon-13 nuclear magnetic resonance in our laboratory. Other chemicals and solvents (analytical grade) were purchased from Xilong Scientific Co., Ltd. (China). The endophytic fungal strain *C. globosum* DX-THS3 (CCTCC M2016005) was isolated from healthy Dongxiang wild rice in a nature reserve in Dongxiang county, Jiangxi province, China in our previous work and collected by Jiangxi Normal University, Nanchang, China.

2.2 Production of GAMG by SSF

Licorice straw was dried for 24 h in a drying oven and then crushed to obtain < 3 mm grain diameter. *C. globosum* DX-THS3 was cultured in a 500 mL flask with 200 mL seed culture medium (potato dextrose broth (PDB)) at 28 °C on a rotary shaker at 150 rpm for seed broth preparation. Then, 2 mL seed broth was cultured in 15 g sterile licorice straw grain, which was pre-added with 5 mL sterile water at 28 °C for the production of GAMG. After 20 days of culture, 2 g culture medium was ground and added to 100 mL water for the determination of GAMG using thin layer chromatography (TLC) and ultraperformance liquid chromatography (UPLC, Waters, USA).

2.3 Optimization of SSF conditions

Strain DX-THS3 was cultivated in different fermentation conditions to detect the GUS activity and yield of GAMG for optimization of fermentation conditions. First, 2 mL seed broth was cultivated in licorice straw of different particle sizes (grain diameter: 0–0.25, 0.26–0.85, and 0.86–3.0 mm) at 28 °C. Then, the GUS activity and yield of GAMG were analyzed after culturing for 20 days. Then, strain DX-THS3 was grown on licorice straw at 24 °C, 28 °C, and 33 °C for the analysis of GUS activity and yield of GAMG after 20 days. For optimization of seed age, different seed broths cultivated in PDB medium for 48, 60, 72, 84, 96, 108, 120, and 132 h were grown on licorice straw at 28 °C to analyze the GUS activity and yield of GAMA after culturing for 20 days. To optimize the inoculum of seed broth, different volumes of seed broths (15%, 20%, 25%, 30%, 35%, and 40%, v/w) in licorice straw at 28 °C to analyze the GUS activity and yield of GAMA after culturing for 20 days. To optimize the water content of licorice straw, strain DX-THS3 were cultured on licorice straw containing different water contents (straw: water = 1:2, 1:2.5, 1:3, 1:3.5, m/m) at 28 °C to analyze the GUS activity and yield of GAMA after 20 days of culture. Finally, glucose, fructose, sucrose, lactose, NH₄NO₃, peptone, yeast powder, and yeast extract were added to the licorice straw for optimization of carbon and nitrogen sources. All cultivations were performed in triplicate.

2.4 Determination of lignocellulosic enzyme activities

Two grams of solid-state medium was sampled, ground by liquid nitrogen, and then dissolved in 15 mL sodium acetate (pH 5.0). The suspension was centrifuged at 4 °C and 10,000 × g, and the supernatant was collected for the preparation of crude enzymes. Endoglucanase activity (CMCase) was assayed in a reaction containing 1 mL carboxymethyl cellulose solution (2% sodium acetate, pH5.0, v/v) and 1 mL crude enzymatic solution. After incubation for 30 min at 50 °C, reducing sugar was detected by 3,5-dinitrosalicylic acid (DNS) method (Zhao et al., 2008) with glucose as the standard. FPase activity was analyzed using Whatman No. 1 filter paper (1 × 6 cm², 50 mg) in a 2 mL total volume reaction containing 1 mL crude enzymatic solution and 1 mL sodium acetate (pH 5.0) for 60 min at 50 °C. Then, the reducing sugar was detected by DNS method. One unit (U) of CMCase and FPase activity was defined as the amount of enzyme that released 1 μmol glucose equivalent per minute. Xylanase activity was assayed in a solution containing 1% (w/v) xylan (1 mL) in 50 mM solidum acetate buffer (pH 5.0) and appropriately diluted crude enzyme (1 mL). After 10 min incubation at 50 °C, reducing sugar was detected by DNS method. One unit (U) of xylanase activity was defined as the amount of enzyme that released 1 μmol xylose equivalents per minute. β-Glucosidase activity was detected based on the release of p-nitrophenol (pNP) from the p-nitrophenyl-β-d-glucopyranoside (pNPG) substrate, and the absorbance was read at 430

nm. The assayed reaction containing 1 mL crude enzyme and 1 mL pNPG (5 mM) was incubated for 10 min at 50 °C and then disrupted by 3 mL 0.5 M sodium carbonate. One unit (U) of β -glucosidase activity was defined as the amount of enzyme that released 1 μ mol pNP equivalent per minute.

2.5 Determination of total and reducing sugars

Two grams of solid-state medium was sampled, ground by liquid nitrogen, and then dissolved in 15 mL water. The suspension was centrifuged at 4 °C and 10,000 \times g, and the supernatant was collected for sample preparation. The total sugar of solid-state medium was determined in accordance with the phenol sulfuric acid method (Masuko et al., 2005), whereas reducing sugars were quantified by the DNS method.

2.6 Determination of GUS activity

Two grams of solid-state medium was sampled, ground by liquid nitrogen, and then dissolved in 15 mL sodium acetate (pH 6.0). The suspension was centrifuged at 4 °C and 10,000 \times g, and the supernatant was collected for the preparation of crude enzymes. GUS activity was assayed by the reaction containing 200 μ L crude enzymatic solution and 800 μ L GL solution (2 g/L). Then, the reaction was incubated at 45 °C for 1 h and disrupted by boiling water and analyzed by UPLC. One unit (U) of GUS activity was defined as the amount of enzyme that released 0.1 μ mol GAMG equivalent per minute.

2.7 Determination of product and yield

Two grams of solid-state medium was sampled, ground by liquid nitrogen, and dissolved in 15 mL water. The suspension was centrifuged at 4 °C and 10 000 \times g, and the supernatant was collected and measured with a metered volume of 100 mL for sample preparation. Then, GAMG and GA were determined by UPLC. UPLC analysis was performed on an ACQUITY UPLC (Waters, USA) instrument using a C18 column (4.6 mm \times 250 mm, 5 μ m, InertSustain, Japan) and UV detector, at the detection wavelength of 254 nm, injection volume of 10 μ L, flow rate of 1.0 mL/min, and mobile phase MeOH–0.5% acetate (80:20).

The concentration of GAMG in samples (defined as C_s (mg/mL)) was determined by UPLC with a standard curve. The concentration of GAMG per gram of solid-state medium (defined as C_{ssf} (mg/g)) was detected by $C_{ssf} = (C_s \times 100)/2$. The production of GAMG by SSF (defined as Y (mg/g)) was quantified by $Y = C_{ssf} \times m/m_0$, whereas m_0 is the weight (g) of initial substrate (licorice straw, 15 g in this study), and m is the weight (g) of solid-state medium after fermentation. The productivity of GAMG by SSF (defined as P (mg/g/day)) was shown by $P = Y/\text{day}$.

3. Results And Discussion

3.1 Production of GAMG by *C. globosum* DX-THS3 using licorice straw as substrate

C. globosum DX-THS3 is an endophytic fungus, and it was isolated from Dongxiang wild rice in our previous work (Wang et al., 2015b). In this study, strain DX-THS3 can utilize licorice straw as a substrate for producing GAMG. Strain DX-THS3 was cultivated in licorice straw to produce GAMG by SSF (Fig. 1A). After 20 days of cultivation, *C. globosum* DX-THS3 covered almost all of the licorice straw (Fig. 1A, **right**). Solid-state medium was sampled, and the product was analyzed by TLC to detect the GAMG. As shown in Fig. 1B, only GL was detected in the licorice straw (b), but after 20 days of fermentation, considerable GAMG was detected using TLC (c). UPLC was performed for identification and further confirmation of these products. Our results confirmed that GAMG was the product in SSF (Fig. 1C). Thus, our results show that GL of licorice straw can be bio-transformed to produce GAMG using licorice straw as a substrate by *C. globosum* DX-THS3.

GAMG is an innovative functional sweetener with higher sweetness and stronger pharmacological activity than GL (Lin et al., 2009; Li et al., 2017; Mizutani et al., 1994). Biocatalysis of GAMG is a more environment-friendly and efficient than the chemical method (Brieskorn & Lang, 1978). To date, several microorganisms (mainly fungi) are screened for GL transformation to produce GAMG. However, a limited number of microorganisms have a GL-hydrolyzing ability. Especially, microorganisms that can selectively transform GL to generate GAMG are rarely found. The previously reported microorganisms with GL-hydrolyzing ability mainly include fungi, such as the filamentous fungus *P. purpurogenum* Li-3 (Zou et al., 2013) and *Talaromyces pinophilus* Li-93 (Xu et al., 2018), and screened from soil. However, the poor substrate specificity and low efficiency are the main disadvantages that limit the further application of these reported fungi. On the other hand, the production of GAMG by microorganic enzymes (GUS) involve tedious steps, including strain cultivation, fermentation, enzyme extraction, enzymatic reaction, and product separation, that seriously limit the application of microorganic enzymes in industries. Thus, screening microorganism from other sources and a novel strategy for high-efficient utilization of microorganic enzymes should be suggested. In this study, an endophytic fungi *C. globosum* DX-THS3 selectively and efficiently transformed GL to produce GAMG when using licorice straw as substrate in SSF. To our knowledge, this work is the first to report the GL production by SSF using endophytic fungi. This research also contributes to the application of endophytic fungi as potential industrial strains in food, biopharmaceutical, and biotechnological industries.

3.2 Optimization of SSF conditions for GAMG production by C. globosum DX-THS3 using licorice straw as substrate

We optimized the SSF conditions for GAMG production by *C. globosum* DX-THS3 using licorice straw as substrate in the present study. The particle size of the substrate is one of the key factors in SSF. Thus, we first investigated the effect of particle size of licorice straw on GAMG production by *C. globosum* DX-THS3. As shown in Fig. 2A, 4.56 mg/g (yield, Y) GAMG was produced by *C. globosum* DX-THS3 when using small-sized licorice straw (0–0.25 mm) as the substrate after 20 days of fermentation; 10.47 mg/g (Y) of GAMG was obtained using a medium particle size (0.26–0.85 mm) of licorice straw after 20 days of fermentation, and 10.35 mg/g (Y) GAMG was detected using licorice straw with a large particle size (0.86–3.0 mm). Our results show that the medium particle size of licorice straw was better than the other

particle sizes for the production of GAMG by *C. globosum* DX-THS3. Furthermore, other fermentation conditions, including temperature, seed age, inoculum size, and solid–liquid ratio (substrate:water, m/v), were optimized. Our results show that 28 °C (Y = 10.47 mg/g, Fig. 2B), 96 h seed age (Y = 13.46 mg/g, Fig. 2C), 20% inoculum size (Y = 19.78 mg/g, v/w, Fig. 2D), and 1:3 solid–liquid ratio (Y = 11.75 mg/g, Fig. 2E) were the optimal conditions for the production of GAMG by SSF using *C. globosum* DX-THS3. These optimal SSF conditions (0.26–0.85 mm licorice straw, 28 °C, 96 h seed age, 20% inoculum size, and 1:3 solid–liquid ratio) were confirmed.

The fermentation conditions can markedly influence the products (not only the kinds but also the yields) when fermenting using microorganisms (Singh et al., 2017). Similar to liquid fermentation, temperature and seed age should be considered when using microorganisms (most of which belong to fungi) for SSF. However, special fermentation conditions, such as the particle size of substrate and water content, should be primarily considered. In general, oversized or extremely small particle size of substrate is unsuitable for SSF using fungi. Oversized particles can affect substrate release, especially that of lignocellulose, and significantly reduce the contact area of fungi with the substrate (Pandey, 2003). On the other hand, an extremely small particle size can influence fungal growth (Pandey, 2003). Water and substrate powder will mix to form a tight bulk or pellet, which not only significantly reduces oxygen transfer but also obstructs in-depth growth of fungal mycelium. In this study, the particle size of licorice straw was first optimized, and our results showed a suitable particle size of 0.26–0.85 mm (Fig. 2A) for the production of GAMG by SSF using *C. globosum* DX-THS3. Water content of substrate is another important factor for SSF by fungi. Low water content is a poor condition for fungal growth. However, a high water content can also inhibit fungal growth, that is, when oxygen transfer is obstructed by high water content of substrate. Thus, suitable water content is required in SSF, and a similar result was obtained in our study (Fig. 2E).

3.3 High activity of lignocellulosic enzymes during the degradation of licorice straw by *C. globosum* DX-THS3

C. globosum DX-THS3 was first cultured in pre-treated licorice straw under the above fermentation conditions to further investigate the feasibility of this novel strategy for GAMG production. *C. globosum* DX-THS3 mycelium was grown slowly in the early stage of fermentation (0–6 days). Then, the growth of *C. globosum* DX-THS3 gradually hastened in the middle stage of fermentation (7–12 days), and the mycelium covered the licorice straw after 20 days of fermentation. Then, deep fermentation was performed (Fig. 3A). The yields of GAMG by *C. globosum* DX-THS3 using licorice straw as substrate were analyzed during SSF to detect the production of GAMG by *C. globosum* DX-THS3. The production of GAMG was extremely low in the early and middle stages (0–18 days) of SFF (Fig. 3B). Until 18 days of fermentation, the yield of GAMG significantly increased. Our results demonstrate that the yield of GAMG reached 13.73 mg/g after 20 days, and the percent conversion of GL reached 90% (Y = 31.5 mg/g) after about 33 days of SSF (Fig. 3B). Furthermore, the total and reducing sugars were detected during SSF to investigate the utilization of carbon source by *C. globosum* DX-THS3. The total sugar of substrate continuously decreased during 0–30 days of SSF and stabilized afterward (Fig. 3C). Thus, the growth period of *C. globosum* DX-THS3 was mainly at 0–30 days of SSF. The reducing sugar increased rapidly

at the early stage of SSF (0–7 days), and the highest concentration was detected at 7 days. Then, the reducing sugar was largely utilized by *C. globosum* DX-THS3 (Fig. 3C). The reducing sugar lowly increased again at the middle stage of SSF (20–22 days, Fig. 3C). The corresponding enzymatic activities, including those of CMCase, FPase, β -glucosidase, and xylanase, were analyzed during SSF to investigate the variation in reducing sugar during SSF by *C. globosum* DX-THS3. Lignocellulosic enzymatic activities were observed at the early stage of SSF, and the highest activities of CMCase (29.25 U/g), FPase (232.5 U/g), and xylanase (72.52 U/g) were detected at 10, 7, and 7 days of SSF, respectively (Table 1 and Fig. 3D). Meanwhile, our results showed rare β -glucosidase activity during SSF of *C. globosum* DX-THS3, whereas 6.42 U/g enzymatic activity was detected at 22 days of SSF.

Table 1
Analysis of lignocellulose-degrading enzymatic activities and production of GAMG under optimal fermentation conditions.

	Enzymes	Max enzymatic activity (U/g)	Days (d)
Non-optimization	CMCase	29.25 ± 1.05	10
	FPase	232.5 ± 30	7
	β -glucosidase	6.42 ± 0.5175	22
	Xylanase	72.52 ± 4.00	7
	GUSase	264 ± 12	20
Optimization	CMCase	33.67 ± 2.48	5
	FPase	245.8 ± 13.4	3
	β -glucosidase	5.78 ± 0.69	20
	Xylanase	83.44 ± 3.76	3
	GUSase	271.42 ± 6.54	10

Biotransformation has more potential than chemical approaches because of its high yield, high selectivity, and environmental compatibility. Compared with liquid fermentation, SSF has many advantages, including low cost and simple processing, for the production of certain compounds. Meanwhile, excellent lignocellulose-degrading activities for utilization of complex substrates (usually crop straw) by microorganisms are needed. Thus, fungi that harbor rich genes coding lignocellulosic enzymes and excellent lignocellulose-degrading activities are usually used in SSF for the production of certain compounds in most previous reports; such fungi include *Ceratocystis paradoxa* TT1 (Nutongkaew et al., 2019) and *Trichoderma koningiopsis* TM3 (Nutongkaew et al., 2019) for the degradation of oil palm trunk to produce reducing sugar. *Trichoderma asperellum* UC1 (Ezeilo et al., 2019) utilizes raw oil palm frond leaves to produce cellulase and xylanase. Similarly, high lignocellulose-degrading enzymatic activities were found during SSF (Table 1 and Fig. 3D) using *C. globosum* DX-THS3 (Table 2). The 234.6 U/g FPase activity was detected during SSF, and this value is substantially higher than the other reported

FPase activities in SSF using fungi. *T. asperellum* UC1 (Ezeilo et al., 2019) showed a relative high FPase (26.02 U/g) activity when using oil palm frond leaves as substrate for SSF, and most FPase activity was detected at 0.09–5 U/g (Table 2). High FPase activity indicates that complex cellulose can be degraded to generate easily hydrolyzed cello-oligomers, which are then further utilized by fungi. Furthermore, compared with the results of other studies, lignocellulose-degrading enzymes, including CMCase, xylanase, and β -glucosidase, from *C. globosum* DX-THS3 during SSF exhibited relative better enzymatic activities (Tables 1 and 2). Thus, high FPase activity and relative excellent CMCase, xylanase, and β -glucosidase activities were detected during SSF by *C. globosum* DX-THS3, demonstrating their wide application in SSF. Our results also strongly consider *C. globosum* DX-THS3 as a potential producer for producing lignocellulose-degrading enzymes.

Table 2

Comparison of CMCase, FPase, β -glucosidase, xylanase, and GUS activities by *C. globosum* DX-THS3 and other fungi under SSF.

Enzymes	Strains	Enzymatic activity (U/g)	Substrate	Reference
CMCase	<i>T. viridae</i> PAJ 01	64.56	Sugarcane bagasse/wheat bran	N.P. Marques <i>et al.</i>
	<i>Chaetomium</i> sp. TCF 01	12.13	Sugarcane bagasse/wheat bran	N.P. Marques <i>et al.</i>
	<i>A. fumigatus</i>	16.90	Wheat straw	A. Shenef <i>et al.</i>
	<i>Botryosphaeria</i> sp.	8.13	Empty fruit bunch	E. K. Bahrin <i>et al.</i>
	<i>Fomitopsis</i> sp. RCK2010	71.70	Wheat bran	D. Deswal <i>et al.</i>
	<i>Hypocreanigrigans</i> TT2	6.10	Oil palm trunk	Nutongkaew T. <i>et al.</i>
	<i>T. koningiopsis</i> TM3	7.13	Oil palm trunk	Nutongkaew T. <i>et al.</i>
	<i>T. asperellum</i> RCK2011	10.25	Wheat bran	Raghuwanshi <i>et al.</i>
	<i>T. asperellum</i> UC1	136.12	Oil palm frond leaves	Ezeilo, U. R. <i>et al.</i>
	<i>C. globosum</i> DX-THS3	29.25	Licorice straw	This work
FPase	<i>Chaetomium</i> sp. TCF 01	0.09	Sugarcane bagasse/wheat bran	N.P. Marques <i>et al.</i>
	<i>A. fumigates</i>	0.98	Wheat straw	Sherief, A. A. <i>et al.</i>
	<i>A. tubingensis</i> NKBP-55	3.8	Copra meal	Prajapati <i>et al.</i>
	<i>Botryosphaeria</i> sp.	3.30	Empty fruit bunch	Bahrin E.K. <i>et al.</i>
	<i>C. paradoxa</i> TT1	1.64	Oil palm trunk	Nutongkaew T. <i>et al.</i>
	<i>Fomitopsis</i> sp. RCK2010	3.50	Wheat bran	Deswal, D. <i>et al.</i>

a: GUS proteins were purified and enriched, and enzymatic activities were detected;

b: GUS activity of solid-state medium was detected.

Enzymes	Strains	Enzymatic activity (U/g)	Substrate	Reference
	<i>T. auraticus</i>	4.40	Wheat straw	Kalogeris E. <i>et al.</i>
	<i>T. asperellum</i> MR 1	0.72	Pressed oil palm petiole fiber	Ikubar M.R.M. <i>et al.</i>
	<i>T. asperellum</i> UC1	26.02	Oil palm frond leaves	Ezeilo, U. R. <i>et al.</i>
	<i>C. globosum</i> DX-THS3	234.6	Licorice straw	This work
β-glucosidase	<i>Chaetomium</i> sp. TCF 01	3.81	Sugarcane bagasse/wheat bran	N.P. Marques <i>et al.</i>
	<i>A. tubingensis</i> NKBP-55	71.0	Copra meal	Prajapati <i>et al.</i>
	<i>I. obliquus</i>	2.58	Wheat bran	Xu X. <i>et al.</i>
	<i>T. asperellum</i> MR 1	0.43	Pressed oil palm petiole fiber	Ikubar M.R.M. <i>et al.</i>
	<i>T. asperellum</i> UC1	130.09	Oil palm frond leaves	Ezeilo, U. R. <i>et al.</i>
	<i>C. globosum</i> DX-THS3	6.42	Licorice straw	This work
Xylanase	<i>T. viridae</i> PAJ 01	351.74	Sugarcane bagasse/wheat bran	N.P. Marques <i>et al.</i>
	<i>Chaetomium</i> sp. TCF 01	39.75	Sugarcane bagasse/wheat bran	N.P. Marques <i>et al.</i>
	<i>A.niger</i> USM AI 1	35.00	Palm kernel cake	Kheng P.P. <i>et al.</i>
	<i>A. fumigatus</i>	56.40	Wheat straw	Sherief A. <i>et al.</i>
	<i>A. tubingensis</i> TSIP9	59.30	Empty fruit bunch	Kitcha S. <i>et al.</i>
	<i>A. tubingensis</i> NKBP-55	167	Copra meal	Prajapati <i>et al.</i>
	<i>T. koningiopsis</i> TM3	56.46	Oil palm trunk	Nutongkaew T. <i>et al.</i>
	<i>T. asperellum</i> MR 1	5.69	Pressed oil palm petiole fiber	Ikubar M.R.M. <i>et al.</i>
a: GUS proteins were purified and enriched, and enzymatic activities were detected;				
b: GUS activity of solid-state medium was detected.				

Enzymes	Strains	Enzymatic activity (U/g)	Substrate	Reference
	<i>T. asperellum</i> UC1	255.01	Oil palm frond leaves	Ezeilo, U. R. <i>et al.</i>
	<i>C. globosum</i> DX-THS3	72.52	Licorice straw	This work
GUSase	<i>A. terreus</i> Li-20	1.86a	–	Xu Y. <i>et al.</i>
	<i>Streptococcus</i> LJ-22	0.77a	–	Park H. Y. <i>et al.</i>
	<i>P. purpurogenum.</i> Li-3	5.90 ×10 ⁴ a	–	Zou S. <i>et al.</i>
	<i>C. globosum</i> DX-THS3	264.17b	Licorice straw	This work
a: GUS proteins were purified and enriched, and enzymatic activities were detected;				
b: GUS activity of solid-state medium was detected.				

3.4 Fructose can significantly improve GAMG production by *C. globosum* DX-THS3

Nitrogen and carbon sources play key roles for production of specific products by fermentation using microorganism. Additional nitrogen and carbon sources were added to the licorice straw to further increase the productivity of GAMG by using SSF. First, the nitrogen source was optimized for GAMG production by SSF using *C. globosum* DX-THS3. NH₄NO₃, peptone, yeast powder, and yeast extract were used as nitrogen sources for the production of GAMG by *C. globosum* DX-THS3 (S Fig. 1A). Our results also show that the yield of GAMG was 1.44- (20.21 mg/g) and 1.19-fold (16.79 mg/g) higher than those of the control (14.02 mg/g) when using NH₄NO₃ and yeast extract as nitrogen sources after 20 days of fermentation, respectively. The GAMG yields were lower than that of the control when using peptone and yeast powder as nitrogen sources after 20 days of fermentation, with values reaching 13.17 and 13.37 mg/g, respectively. We further detected the GUS activity after adding NH₄NO₃, peptone, yeast powder, and yeast extract as nitrogen sources to the medium. Our results also showed the higher GUS activity when using NH₄NO₃ and yeast extract as nitrogen source than the control, whereas those obtained with peptone and yeast powder were lower after 20 days of fermentation (S Fig. 1B). Thus, NH₄NO₃ as additional nitrogen source can increase the yield of GAMG by *C. globosum* DX-THS3 in SSF. Meanwhile, the carbon source for GAMG production was investigated (S Fig. 2). The addition of fructose and glucose can produce 18.38 and 17.12 mg/g of GAMG after 20 days of fermentation (S Fig. 2A and 2B), respectively, which denoted increases of 33.9% and 24.7% than those obtained without a carbon source (13.73 mg/g). The addition of sucrose can generate 13.98 mg/g GAMG but showed no significant effect on the production of GAMG using *C. globosum* DX-THS3 (S Fig. 2C). Our results showed the significant inhibition of GAMG production when adding lactose to the medium for SSF using *C. globosum* DX-THS3

after 20 days of fermentation. A total of 9.23 mg/g GAMG was produced, which was a 34% reduction in GAMG compared with that obtained without a carbon source (**S** Fig. 2D). Furthermore, GUS activities with the addition of carbon sources were further detected after 20 days of SSF. Our results showed similarity to those of GAMG production with carbon source (**S** Fig. 2E). GUS activity with the addition of fructose was 338 U/g, which was significantly higher than that without carbon source (167 U/g). GUS activity with the addition of glucose was 1.5-fold (250 U/g, **S** Fig. 2F) higher than that without a carbon source (control, 167 U/g). Compared with the control, the addition of sucrose showed no significant effect on GUS activity (155 U/g, **S** Fig. 2G), but the addition of lactose significantly inhibited GUS activity (132 U/g, **S** Fig. 2H). Thus, NH_4NO_3 and fructose can significantly promote GAMG production of *C. globosum* DX-THS3 by SSF using licorice straw as a medium.

The yield of GAMG during the SSF period with or without carbon and nitrogen sources was detected to further investigate the production of GAMG using *C. globosum* DX-THS3. First, we detected the yield of GAMG with or without the addition of carbon source (Fig. 4A). The variation trends of GAMG yields were similar. All the test yields of GAMG slowly increased at the initial stage of SSF (0–15 days), whereas those at the middle stage rapidly increased (15–30 days). Then, the yields of GAMG stabilized at about 32 mg/g at the late stage of SSF (after 30 days). Although all the test yields of GAMG exhibited no significant difference at the later stage of SSF (**S** Fig. 3), the productivities of GAMG by SSF with different carbon sources or without a carbon source presented significant differences. Compared with the control (33 days), the addition of fructose was the fastest for GAMG production, and 25 days of SSF was used to reach 90% conversion ($Y = 31.52 \text{ mg/g}$), 28 days for the addition of glucose, and 30 days for the addition of sucrose. The addition of lactose to the medium can inhibit GAMG production by *C. globosum* DX-THS3 in SSF, requiring 38 days to reach 90% conversion. Meanwhile, the addition of nitrogen source also showed significant effect on the production of GAMG by SSF using *C. globosum* DX-THS3. As shown in Fig. 4B, compared with that without nitrogen source, the addition of NH_4NO_3 to the medium achieved improved production of GAMG by *C. globosum* DX-THS3, with a reduction of 9 days in the production period to transform 90% of GL compared with that without a nitrogen source. Yeast extract showed no effect on the production of GAMG, whereas yeast powder and peptone can inhibit GAMG production. Thus, NH_4NO_3 and fructose were further optimized. First, different concentrations of NH_4NO_3 and fructose (3, 5, 7, 9, and 11 mg/g) were added to the SSF medium. Then, GAMG production was detected after 20 days of SSF. Our results show that 7 mg/g NH_4NO_3 and 5 mg/g fructose were the optimum conditions for GAMG production by *C. globosum* DX-THS3 using SSF (Fig. 4C and 4D, respectively). Based on the above results, GAMG was produced faster under these optimal conditions than the control: 0.26–0.85 mm particle size, 28 °C, 96 h seed age, 20% inoculum size, 1:3 solid–liquid ratio, 7 mg/g NH_4NO_3 , and 5 mg/g fructose (Fig. 4E). Under these optimal conditions, the percent conversion of GL reached 90% within 15 days, whereas the control needed 35 days, that is, an additional 20 days needed for 90% conversion (Table 1 **and** Fig. 4E). The productivity of optimization ($P = 2.1 \text{ mg}\cdot\text{g}^{-1}\cdot\text{day}^{-1}$) increased by 133.33% compared with that in non-optimized conditions ($P = 0.9 \text{ mg}\cdot\text{g}^{-1}\cdot\text{day}^{-1}$).

Microorganic fermentation can be classified into three stages: early, middle, and late stages. In general, low-cost and easily available substrates, such as crop straw, are usually used as medium for SSF using fungi. However, complex and adequate enzymes are needed for the degradation of polysaccharides in these substrates (Prajapati et al., 2018; Deswal et al., 2011; Pandey, 2003; Nutongkaew et al., 2019; Ezeilo et al., 2019). Compared with liquid fermentation, more time is needed for fungal cell growth to secrete a complex enzyme system in SSF. Thus, fast accumulation of biomass is the key for shortening the early stage period and increasing the productivity of SSF. To reduce this period, we first optimized the fermentation conditions, including the particle size, temperature, seed age, inoculum size, and water content. However, the early stage of GAMG production was long (about 25 days) (Fig. 3B). Thus, several nitrogen sources that are easily utilized by microorganisms were considered. Glucose, fructose, and several monosaccharides (popular carbon sources) can be directly entered into glycolysis and TCA cycle for fungal growth (Fig. 5A). Therefore, we considered adding these carbon sources to the medium to increase productivity when using microorganisms for SSF. Our results demonstrate that the early stage period of SSF was reduced by about 10 days when adding fructose and NH_4NO_3 to the medium, thus significantly increasing productivity (Table 1 and Fig. 4E). In this study, lignocellulose and GL of licorice straw were used as carbon sources for *C. globosum* DX-THS3 growth, and high lignocellulose-degrading enzymatic activities were detected during 5–15 days of fermentation (Fig. 3D). High GUS activities were observed at 20–25 days (Fig. 3C), and the concentration of reducing sugar abruptly increased at 20 days of fermentation. These findings demonstrate that lignocellulose was first utilized, followed by GL, by *C. globosum* DX-THS3. Thus, certain carbon sources were added to the medium to reduce the early stage period and increase the GAMG production. In summary, at the initial period of SSF, several lignocellulose-degrading enzymes were secreted because of the rare biomass of *C. globosum* DX-THS3. *C. globosum* DX-THS3 slowly grew. Thus, a long period was needed for the accumulation of *C. globosum* DX-THS3 to secrete sufficient lignocellulose-degrading enzymes. Subsequently, GUS was rapidly secreted by *C. globosum* DX-THS3 for the utilization of GL as a carbon source to generate GAMG and glucuronic acid (Gur). If several popular carbon sources, such as fructose, are added to medium, *C. globosum* DX-THS3 will grow fast, which can promote the secretion of lignocellulose-degrading enzymes and fast utilization of lignocellulose, significantly reducing the time for production of GAMG (Fig. 5B). Thus, our study provides a novel, fast, and low-cost method for the production of GAMG.

4. Conclusions

In the present study, *C. globosum* DX-THS3 can use licorice straw as a medium for SSF. After optimization of fermentation conditions, 90% GL was transformed to GAMG within 15 days, whereas the control needed 35 days. The production of GAMG can reach $2.1 \text{ mg}\cdot\text{g}^{-1}\cdot\text{day}^{-1}$, which was 2.33-fold higher than that of the control. Meanwhile, high lignocellulose-degrading enzymatic activities were also detected during SSF. Therefore, our study contributes to the application of SSF in the production of GAMG and strongly demonstrated *C. globosum* DX-THS3 as a potential candidate for producing lignocellulose-degrading enzymes.

Declarations

Authors' contributions

BLG, YWX, QZ, and JRS did the experiments. ZBZ provided resources. DZ and BLG supervised the project, designed the experiments, and wrote the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by the Natural Science Foundation of China (31960078), by Foundation of Jiangxi Educational Committee (GJJ190593, GJJ180636), by Funds of Jiangxi Science & Technology Normal University (2018BSQD028, 2017XJZD004).

Acknowledgements

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have read this article and have approved its submission to *Bioresources and Bioprocessing*.

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Figures

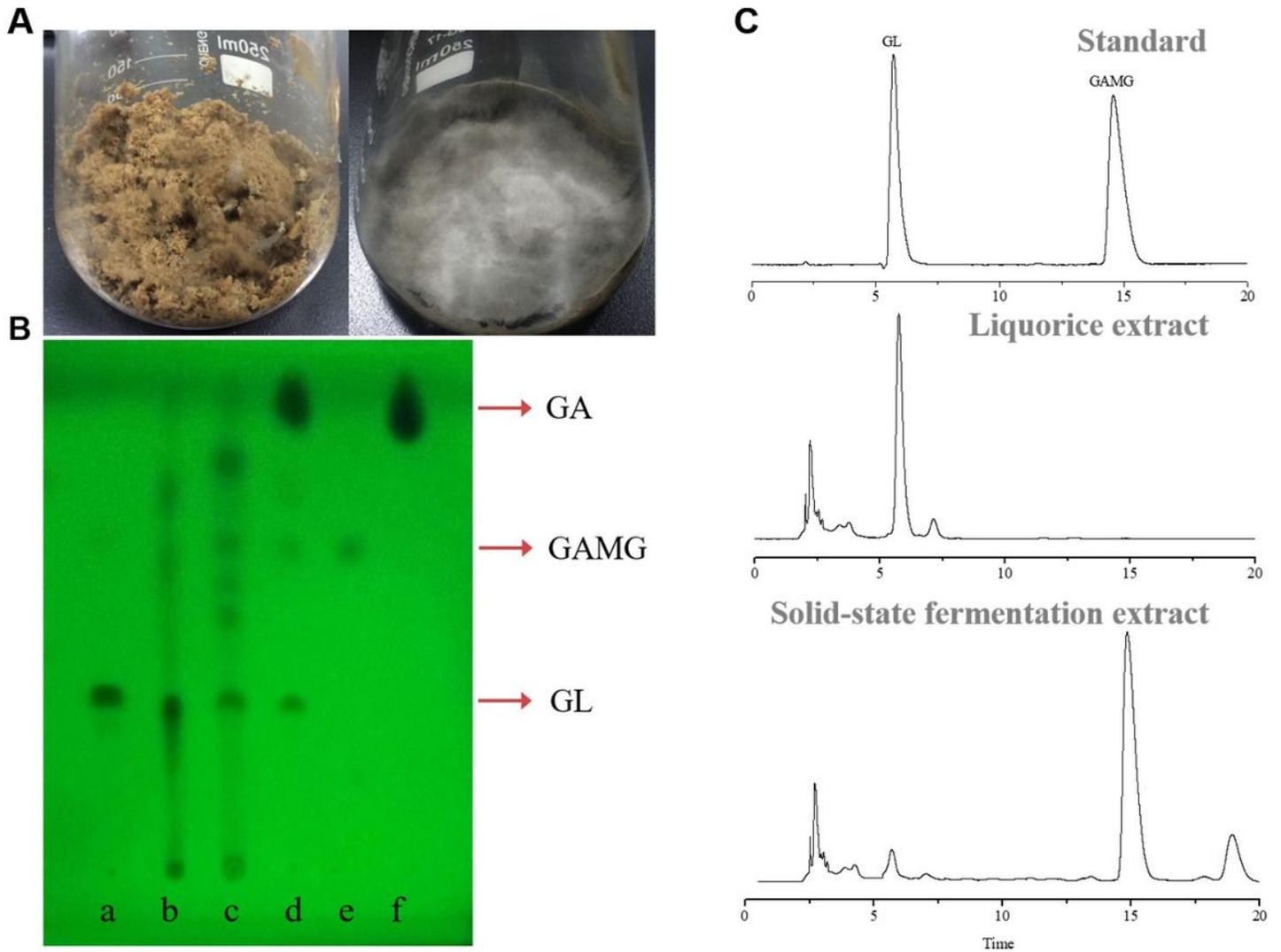


Figure 1

C. globosum DX-THS3 can grow on licorice straw for GAMG production. Licorice straw was used as a medium for *C. globosum* DX-THS3 growth (left, A). Depth fermentation was performed after 20 days (right, B), and the product was analyzed by TLC (B) and UPLC analyses.

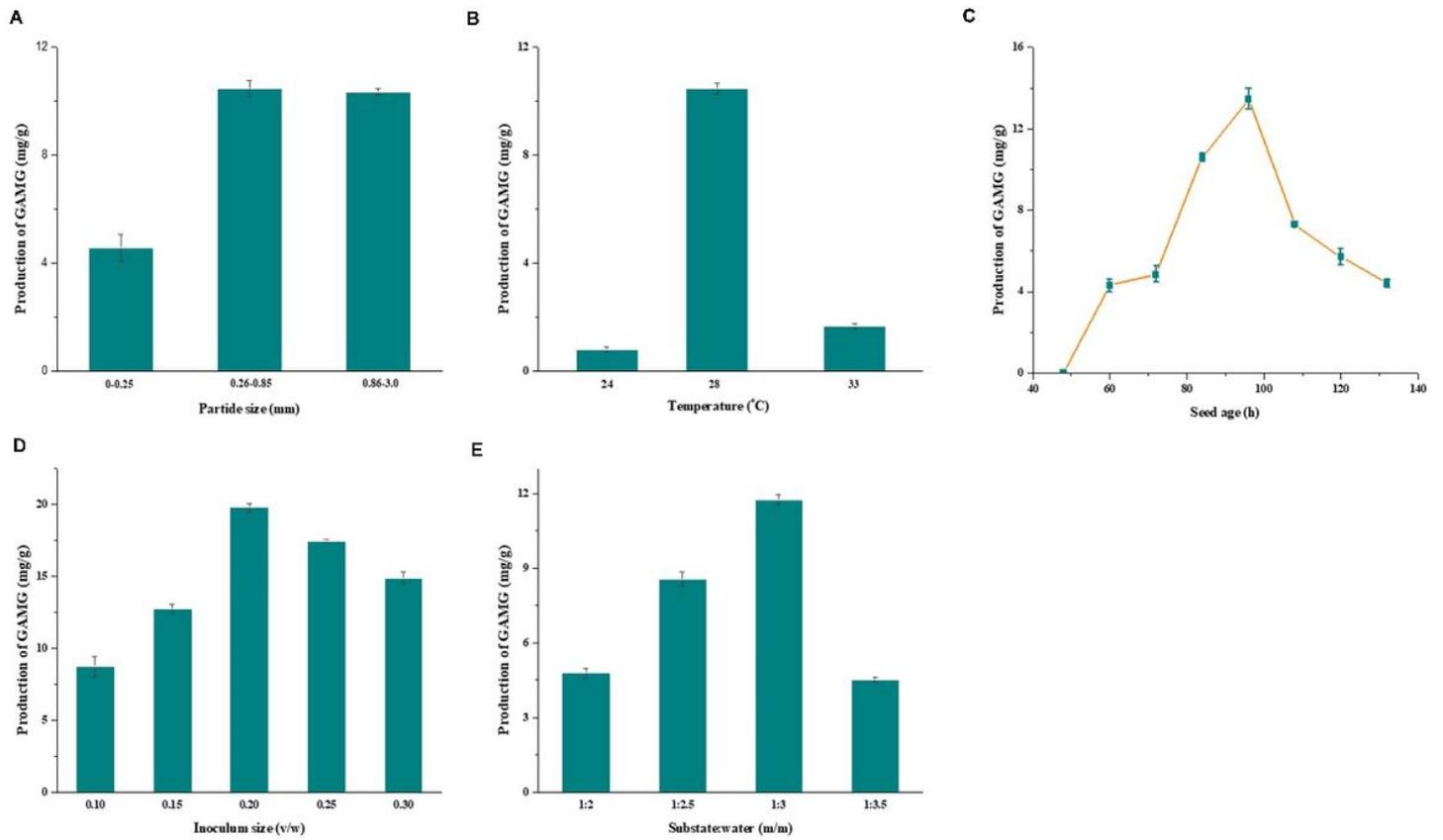


Figure 2

Optimization of fermentation conditions, including particle size (A), temperature (B), seed age (C), inoculum size (D), and substrate:water (E).

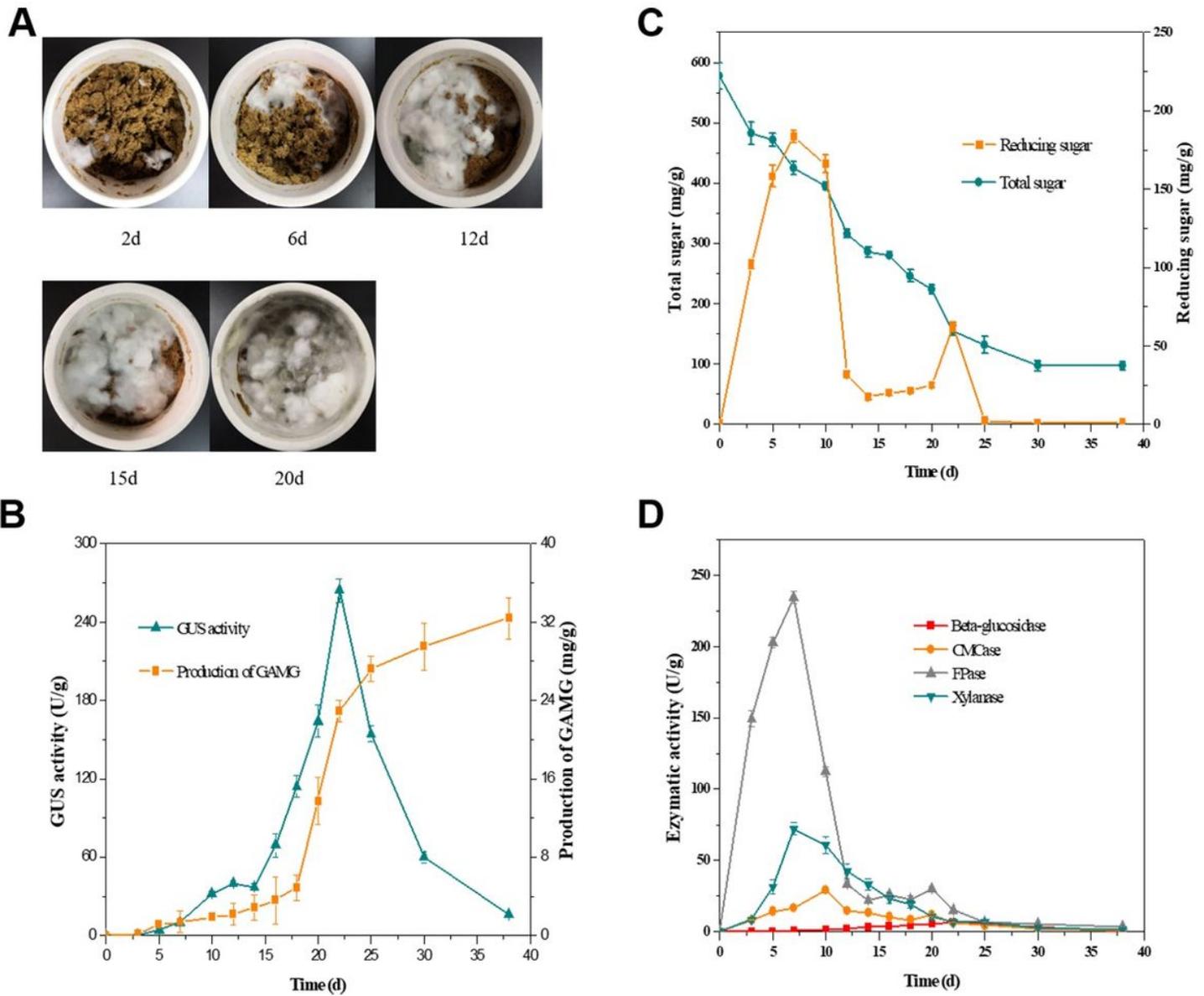


Figure 3

GUS activity, GAMG, reducing sugar, total sugar, and lignocellulose-degrading enzymatic activity profiles of *C. globosum* DX-THS3 under optimal SSF conditions. (A) *C. globosum* DX-THS3 was grown on licorice straw for depth fermentation. (B) GUS activity and yield of GAMG profile; (C) reducing sugar and total sugar profile; (D) CMCase, FPase, β -glucosidase, and xylanase profile.

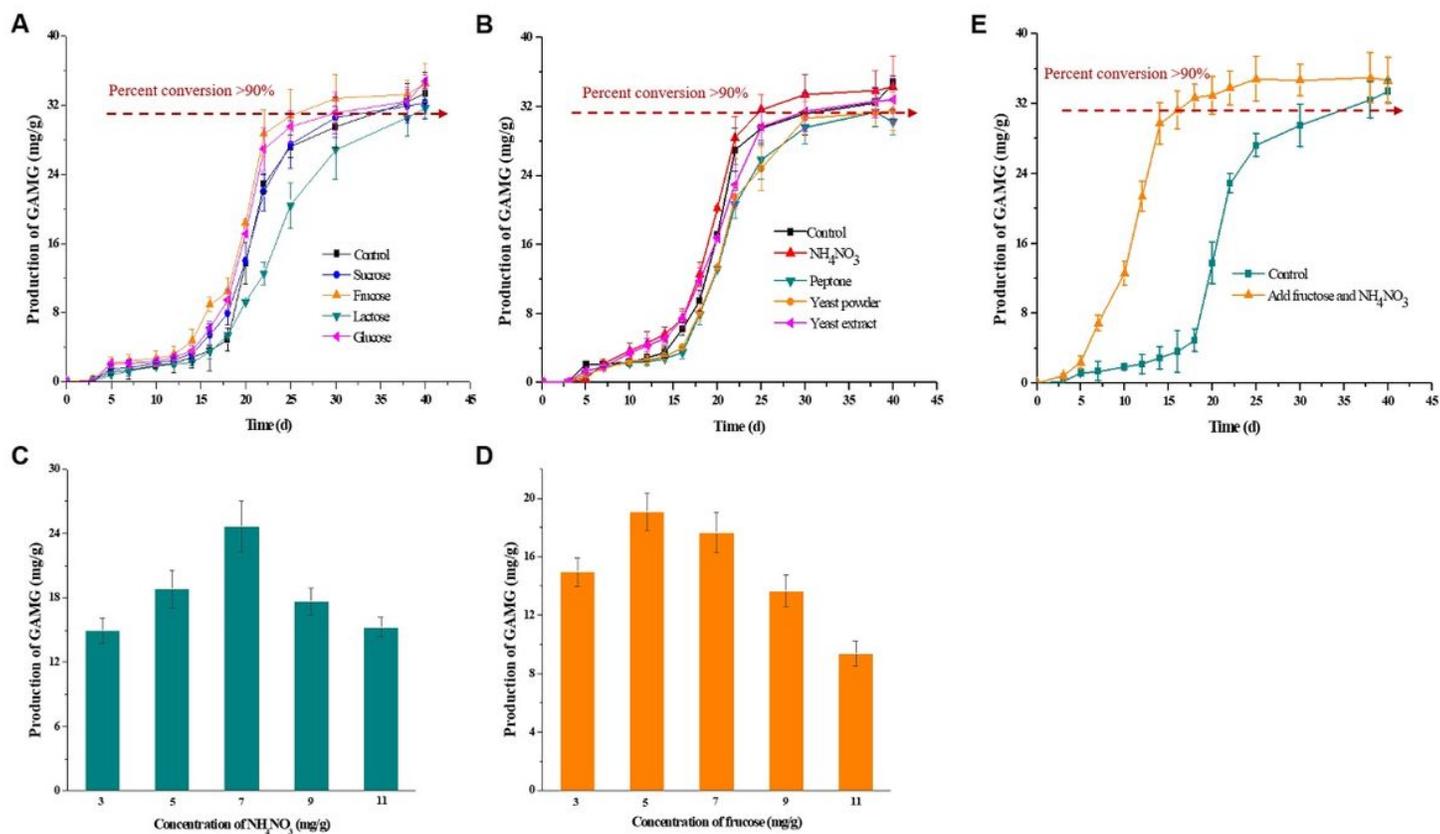


Figure 4

Effect of nitrogen and carbon sources on GAMG production. Yield of GAMG profile with the addition of (A) different nitrogen sources, (B) various carbon sources, (C) different concentration of NH_4NO_3 , (D) different concentration of fructose and (E) under optimal fermentation conditions.

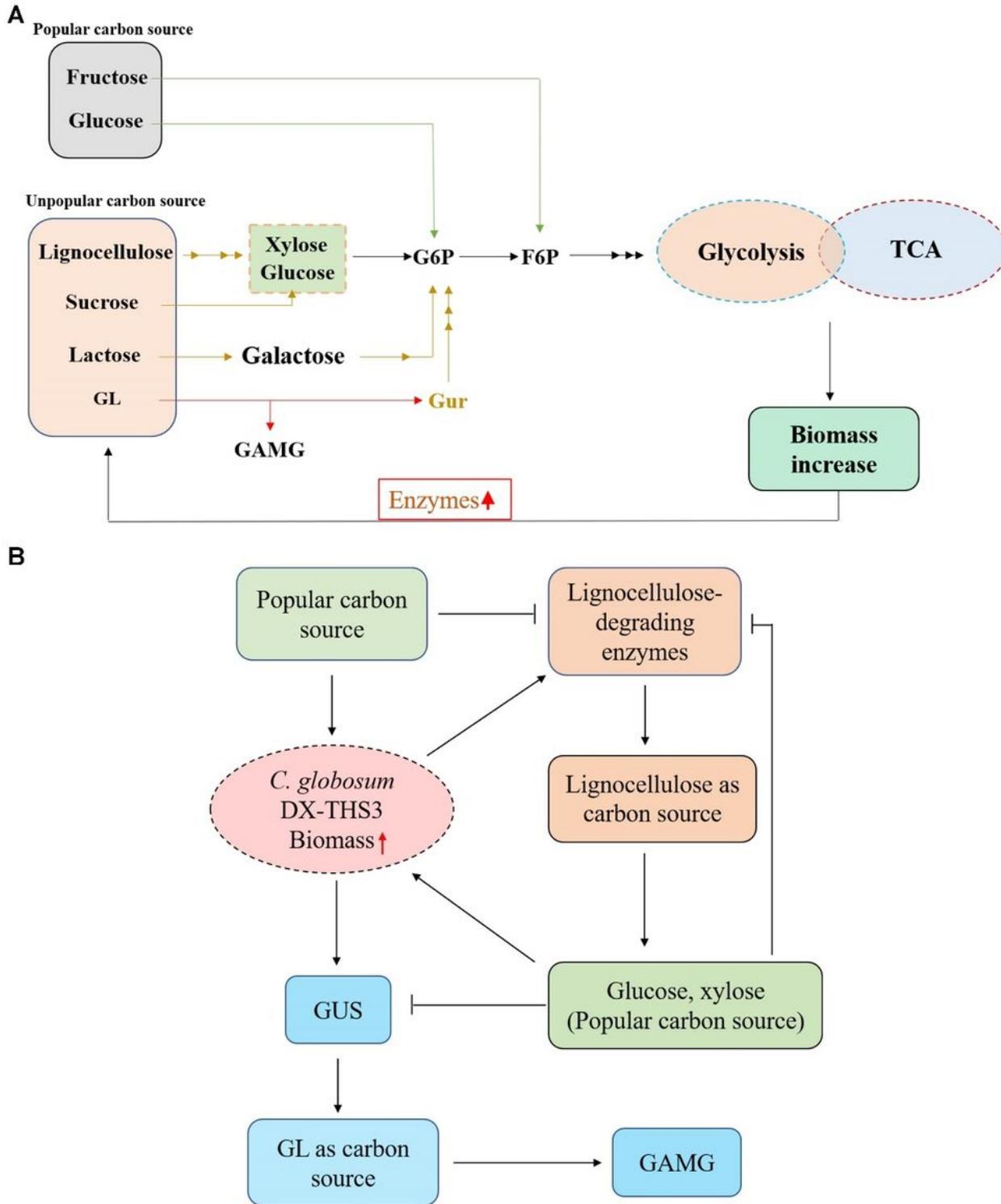


Figure 5

Predicted mechanism for promoting GAMG production by the addition of fructose to the medium. (A) Fructose and glucose (popular carbon sources) were more rapidly utilized by *C. globosum* DX-THS3 than lignocellulose, sucrose, lactose, and GL because such popular carbon sources can directly enter glycolysis and TCA cycle. (B) Popular carbon sources can promote the rapid accumulation of *C.*

globosum DX-THS3 biomass, producing more lignocellulose-degrading enzymes, rapidly utilizing lignocellulose, and finally fast-transforming GL into GAMG.

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