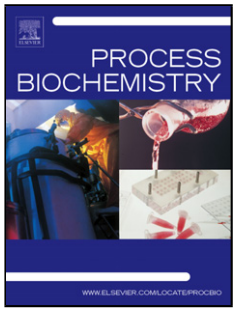

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The Limited Deglucosylation Process of β-Glucosidase in *Bacillus cereus* H62L for Biotransforming Secoisolariciresinol Diglucoside into Mammalian Lignans

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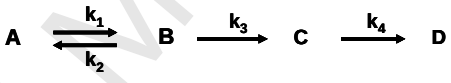
Running title: Biotransformation of secoisolariciresinol diglucoside

Abstract

The amount of major lignans present in the alkaline extract of the defatted flaxseed (in mg/g) is as follows: secoisolariciresinol diglucoside (SDG, 109.80), *p*-coumaric acid glucoside (CAG, 70.21), ferulic acid glucoside (FAG, 68.81), secoisolariciresinol (SECO, 0.49), coumaric acid (CA, 0.37), and ferulic acid (FA, 0.85). When biotransformed, SDG yields two valuable mammalian lignans (MLG): enterodiol (END) and enterolactone (ENL). *Bacillus cereus* strain H62-L1 was isolated and tested for the process of bioconversion. Kinetic analysis revealed the sequential reaction SDG (A)→ CAG (B)→ END (C)→ ENL (D) to be reversible at the first step with the generalized reaction type 

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The obtained rate coefficients were as follows: k_1 = 0.001-0.028 h^{-1}, k_2 = 0.006-0.077 h^{-1}, k_3 = 0.013 h^{-1}, and k_4 =0.002 h^{-1}, and the overall kinetic parameter $k_1/(k_2+k_3)$ varied from 0.05 to 1.32, mostly not favoring the forward deglucosylation reaction. SDG with higher purity favored the forward reaction. In conclusion, to facilitate the production rate, we propose i) using substrate SDG with high purity at a higher concentration; ii) using an allosteric inhibitor to block the reverse reaction from SECO to SDG; or iii) genetically modifying *Bacillus cereus* H62-L1.

Keywords: Flaxseeds; secoisolariciresinol diglucoside; enterodiol; mammalian lignans; glucosidase

1. Introduction

Lignans are produced via the shikimic acid pathway and are categorized as secondary plant metabolites [1,2]. Most of the lignans occur in form of glycosidic conjugates. The total lignan content in flaxseeds constitutes approximately 1.8 % (w/w) [3]. Secoisolariciresinol diglucoside (SDG, $C_{32}H_{46}O_{16}$, MW: 686.7) (**I**), *p*-coumaric acid glucoside (CAG) (**V**), and ferulic acid glucoside (FAG) (**VI**) are the major lignans. Upon ingestion, SDG is deglucosylated by the β-glucosidase present in the intestinal microflora and yields free lignan secoisolariciresinol (SECO, $C_{20}H_{26}O_6$, MW: 362.4) (**II**), which is further consecutively dehydoxylated and demethylated to yield enterodiol (END, $C_{18}H_{22}O_4$, MW: 302.1) (**III**). On dehydration, Compound **III** further produces enterolactone (ENL, $C_{18}H_{18}O_4$, MW: 298.3) (**IV**) [4] (Fig. 1, 2).

Type 2 diabetes is associated with an increase in oxidative stress, and SDG is effective in delaying the development of diabetes [7-9]. The downstream products of SDG, free END and ENL together with their corresponding glucosides, form a category of phenolics popularly named the mammalian lignans (MLG). MLG inhibit the aromatase responsible for the biotransformation of testosterone into estrogens [10].When bound to estrogen receptors α (ERα) and β (ERβ), MLG evoke a strong antagonistic effect on estrogen [11,12]. MLG exhibit a diversity of bioactivities involving antioxidative, anti-atherosclerotic, anticancer, and anti-diabetes mellitus effects [8,9,13]. MLG stimulate the production of human sex hormone binding globulin (*h*-SHBG). The latter is able to entrap the free estrogens in serum, which results in reduced serum estrogen levels available for tumor growth [14-16]. MLG

help to modulate α-fetoprotein by reducing the number of estrogen sensitive cells to favor the proliferation of normal cells [17]. The literature also indicates that MLG prevent the prevalence of colon cancer by stimulating glucuronidase production and inhibiting estrogen sulfatase [18,19], and investigations into their mass production are emerging.

Humans and doves typically ingest seasonal flaxseeds as part of their daily diet. Consequently, they can feasibly become the main distributor of flaxseeds in the wild. We hypothesize that human and dove feces may carry potentially active microbial strains. We isolated a strain of *Bacillus cereus* from human feces and performed a serial fermentation process with the aim of finding a novel technology for the mass production of MLG. Beginning with SDG, kinetic analyses were performed to evaluate the biotransformation data.

2. Materials and methods

2.1. Source of flaxseeds

Flaxseed (*Linum usitatissimum* L.), a product imported from the USA, was supplied by the local Tong-Lin Trading Co. in Taichung, City (Taiwan). The samples were preserved at -80^{o}C before treatment.

2.2. Chemicals

Secoisolariciresinol diglucoside (SDG) was a product of Chromadex Co., (USA). Secoisolariciresinol (SECO), enterolactone (ENL) and enterodiol (END) were provided by Fluka Co. (Bern, Switzerland). *p*-coumaric acid (CA), *p*-coumaric acid

glucoside (CAG), ferulic acid (FA), ferulic acid glucoside (FAG), and *p*-nitrophenyl-β-D-glucopyranoside (PNPG) were manufactured by Sigma-Aldrich (St. Louis, MO. USA). Liquid broth (25 g/L) and liquid broth agar (40 g/L) were purchased from Schalau Co. (Spain). Synergi 4 μ Fusion-RP 80A (250×4.6 mm), Sep-Pak Vac 3CC (500 mg) C18 cartridges, Gram's stain solution, potassium hydroxide, liquid broth phosphate buffer solution (PBS) were all supplied by Wako Pure Chemical Inc. (Osaka, Japan). Chromatography grade acetonitrile was supplied by J. T Baker Co. Solution A was a 5% acetonitrile/phosphate solution (pH 2.8). The solution was freshly filtered with a 0.45 μm micropore filter before use or alternatively transferred into a serum bottle and stored at -5^{o}C.

2.3. Preparation of defatted flaxseed flour (DFF)

Fresh flaxseeds were dried in an oven at 50^{o}C for 5 days. The desiccated flaxseeds were blended and pulverized with a grinder, and sieved through a #60 mesh screen. The powder that passed through the mesh #60 was collected and stored at -80^{o}C. Five hundred milliliters of n-hexane was added to 100 g desiccated powder. The mixture was transferred to a 1000 mL serum bottle and heated on a heating plate with continuous magnetic agitation at 60^{o}C for 1 h. The extract was separated by ultracentrifugation at 12000×*g* for 5 min (4^{o}C). The extraction was repeated in triplicate, and the three n-hexane extracts were combined. The n-hexane was removed by evaporation during ventilation under the hood. The wet residue was further dried in a hot, aerated hood at 40^{o}C for 5-6 h until completely dry. The dry powder was packed

in 5 g packs and stored at -80^{o}C for further use (DFF).

2.4. The ethanolic extract of DFF (EEF)

The method of Westcott and Paton [20] was followed to isolate the lignan conjugates from the flaxseeds. Briefly, to 235 g of DFF was measured and transferred into in a 5 L concave Erlenmeyer flask, and 1570 mL of ethanol (70%) was then added to the flask. The maceration was carried out in a shaking (100 rpm) water bath at 55^{o}C for 6 h. The solution was left to stand at ambient temperature to facilitate the separation. The supernatant was decanted into a serum bottle. The bottom layer was centrifuged at 8000×g in a Kubota KS-5200C (Osaka, Japan) at 4^{o}C for 10 min to recover the extract. The supernatants were then combined, and the extraction was repeated with 70% ethanol at 55^{o}C at 100 rpm for 1 h. All the supernatants were combined and evaporated under a reduced pressure to remove the ethanol. Both the evaporated product and the solid residue were lyophilized respectively (King Mech FD-20L-6S, Taipei, Taiwan) [20]. The desiccated residue was designated as the ethanolic extract of DFF (EEF) and stored at -20^{o}C until further use.

2.5. The alkaline hydrolysate of EEF (EEA)

Alkaline hydrolysis of SDG cleaves the ester linkages of the phenolic compounds in flaxseed, e.g., the linkages in the SDG–3-hydroxy-3- methylglutaric acid (HMGA) oligomer. Thus the method of Johnsson et al. [21,22] was followed. Briefly, 10 g of EEF was transferred into a 1 L serum bottle, and 500 mL of 0.5 M NaOH was added to the bottle. The mixture was macerated at ambient temperature at 200 rpm for 1 h, then

adjusted to pH 6.5 with 2 M H_2SO_4 and mixed well. The mixture was ultracentrifuged at 10000×g for 10 min (4°C). The supernatant was reduced to 1/3 of its original volume in a rotary evaporator under reduced pressure. The condensed supernatant was lyophilized, weighed and stored in a sample vial (EEA).

2.6. HPLC analysis

High-performance liquid chromatography (HPLC) analyses were performed using a Shimadzu HPLC system equipped with SPD-10A (UV-Vis) detector and LC-10AT pump (Shimadzu, Japan). The column used was Synergi 4 μ Fusion-RP80A (ℓ×i.d. = 250×4.6 mm) (Phenomenex, Canada). The mobile phase was composed of two components A and B. Solution A was a 5% acetonitrile/0.01 M phosphate buffer solution (pH 2.8), and its method of preparation was described in Section 2.2. Solution B was 100% chromatography grade acetonitrile. For HPLC analyses, 0.01 g EEA was dissolved in 1 mL of methanol and filtered with a 0.45 μm micropore filter. A 20 μL aliquot of the filtrate was used for HPLC analysis. The column was eluted at a flow rate of 1 mL/min according to the programmed protocol given by the manufacturer. Briefly, from 0-30 min, the elution was run with 100% of A; from 30-40 min, with 50% A/50% B, and from 40-45 min, the elution was conducted with isocratic 100% A. Authentic samples, which included secoisolariciresinol diglucoside (SDG), secoisolariciresinol (SECO), enterolactone (ENL), enterodiol (END), *p*-coumaric acid (CA), *p*-coumaric acid glucoside (CAG), ferulic acid (FA), and ferulic acid glucoside (FAG) were used for qualitative and quantitative determinations. Retention time was

used as the informative qualitative analysis. The concentrations of authentic solutions were prepared to contain 0, 20, 40, 80, 120, and 160 μg/mL, and 20 μL of each authentic sample was used for each analysis. Similarly, the fermented fluid sample was filtered through a 0.45 μm micropore filter, and a 20 μL aliquot of the filtrate was injected into the HPLC for analysis. The photodiode array detector was operated at 280 nm, and the peaks were integrated using HP Chemstation software. Quantification was assessed by comparing the integrated area of the sample and the authentic samples covered by each peak in the HPLC spectra. The tests were repeated 6 times to determine its precession and reproducibility. The coefficient of variation was <5% [21,22].

2.7. Preparation of tryptone agar and tryptone broth

Tryptone (2.5 g, Bacto Laboratories, Liverpool, Australia), yeast extract (1.25 g, Bacto), NaCl (2.5 g), agar (3.75 g, Bacto) and 250 mL of water (RO) were combined and agitated with heating at 70^{o}C to facilitate dissolution. The solution was transferred into an Erlenmeyer flask and autoclaved at 121^{o}C for 15 min. The sterilized broth was then poured into petri dishes to make agar plates. LB broth was similarly prepared and sterilized.

2.8. Strain isolation

The DFF was aseptically incorporated at 5% (w/v) into the tryptone broth culture medium and mixed well. One loop of fecal sample was inoculated, and the sample was incubated either aerobically or anaerobically for 7 days. The fermentation fluid was

filtered with 0.45μ micropore filter, and a 20 μL aliquot of the filtrate was subjected to HPLC analysis. Cultures capable of producing SDG and other lignans were selected. The selected cultures were plated onto LA plates and incubated at 37^{o}C for 7 days. The colonies capable of producing lignans as shown in Figure 1 were selected. The isolation procedure was repeated until pure cultures were obtained.

2.8.1. Identification of strain H62-L1 by morphological and biochemical tests

Isolated strain H62-L1, which was from a human source, was sent to the Food Industry Research And Development Institute (Hsinchu, Taiwan) for identification.

2.8.2. Identification of strain H62-L1 by 16S rDNA gene sequence

The identification of the newly isolated strain H62-L1 strain was performed by multiplex arrays according to Song *et al*. [23]. Briefly, the group 16S-23S rDNA and species-specific 16S-23S rRNA intergenic spacer region and its flanking 23S rDNA were selected [23]. This experiment was performed at the Food Industry Research And Development Institute (Hsinchu, Taiwan). The voucher for this service has been submitted to the editor as a supplement.

2.9. Preliminary testing of biotransformation capabilities

To compare the biotransformation capability of the growing and the resting cells, three metabolic biotransformation experiments were conducted. The first one was performed to examine the biotransformation activity of growing cells on DFF (Exp. 1). The second investigated the biotransformation capability of the growing cells on EEF (Exp. 2), and the third tested resting cells on EEF (Exp. 3). In Exp. 1, 8 mL of tryptone

broth and 0.5 mL of fresh growing culture were added to 2 mL of the DFF polymer solution, and the mixture was incubated for 360 h. In Exps. 2 and 3, cells (0.2 mL), either growing cells (Exp. 2) or resting cells (Exp. 3), were added to 0.8 mL EEF solution supplemented with tryptone broth, and the mixtures were incubated respectively for 72 h. Before the incubation period, all three cultures were separately transferred into four 50 mL Hinton flasks, and the incubation period was performed with stirring (150 rpm) at 37^{o}C in an orbital shaking incubator (OS1500; USA). Samplings were performed every 12 h.

2.10. Confirmation of the biotransformation pathway

2.10.1. Fermentation starting with the authentic lignans

Resting cells (0.2 mL) were inoculated into a 0.8mL mixture containing authentic SDG, SECO, END, and ENL (each 27 mg/L). The culture was transferred into a 50 mL Hinton flask and incubated in an orbital shaking incubator (OS1500; USA) with stirring (150 rpm) at 37^{o}C. Samplings were performed every 12 h.

2.10.2. EEA fermentation

To further study the biotransformation pathway, EEA was used as the substrate. Resting cells (0.2 mL) were inoculated into a 0.8mL mixture containing EEA supplemented with tryptone broth.. The culture was transferred into a 50 mL Hinton flask and incubated (orbital shaking incubator OS1500; U.S.A) with stirring (150 rpm) at 37^{o}C. Samplings were taken every 12 h.

2.11. Optimum concentration of resting cells

To determine the optimal concentration of resting cells for biotransformation, resting cells at concentration of 0% to 25% were used. Briefly, resting cells (0.5 mL), tryptone broth (0.0 to 7.5 mL), and PBS (0.01M, pH 7.0; 7.5 to 0.0 mL) were added to 2 mL of EEA to make a final volume of 10 mL, which corresponded to resting cell concentrations of 0.0, 5.0, 10.0, 15.0, 20.0, and 25.0%, respectively. These cultures were then transferred into 50 mL Hinton flasks and incubated at 37^{o}C with striring (150 rpm). Samples were taken every 12 h.

3. Results and discussion

3.1. Yields of DFF and EEF

The recovery of desiccated flaxseed flour after desiccation and blending was 72.6% (DFF). Based on the yield of desiccated flaxseed flour, after being defatted, the recovery rate of DFF was 51%. The ethanol extraction yielded 7.8% of the EEF, which contained mainly oligomers of CAG, FAG, and SDG [24] and polyphenol hydrocinnamic acid glucosides [21,22].

3.2. Phenolic compounds present in EEA

After alkaline hydrolysis, EEF yielded the alkaline hydrolysate of ethanolic extract (EEA), which was highly enriched with the flaxseed polyphenolics. The retention times (RT) obtained from the HPLC analysis were (in min): coumaric acid glucoside (CAG) (14.832); ferulic acid glucoside (FAG) (16.895); secoisolariciresinol diglucoside (SDG) (21.929); coumaric acid (CA) (24.696); ferulic acid (FA) (26.138); and secoisolariciresinol (SECO) (30.108). However, END and ENL were not found in

the EEA. To determine the retention times of END and ENL, we spiked the authentic sample to obtain the enterolactone (ENL) (40.147) and enterodiol (END) (33.782) retention times (Table 1, Fig. 3). The amounts of these six major phenolic compounds that were present in the EEA were (in mg/g): CAG (70.21±4.35), FAG (68.81±4.30), SDG (108.80±5.78), CA (0.37±0.01), FA (0.85± 0.03), and SECO (0.49±0.05). A similar report indicated that SDG contributed 56 mol% to the phenolic constituents, while the CAG, FAG, and HDG contents were much lower, contributing only 23, 15 and 6 mol%, respectively [25].

Chemically, the release of CAG, FAG, and SDG can be achieved by alkaline hydrolysis. In fact, this transformation can also be accomplished by the intestinal anaerobic microflora, which involves *Bacteroides fragilis*, *Clostridium cocleantum*, *Clostridium ramosum*, and *Clostridium sacchaogumia* [4,26-29].

3.3. Identification of the isolated strain

The newly isolated strain H62L-1 shared 99.97% sequence identity with the 16s rDNA of *Bacillus cereus*. The strain was identified as a member of the *Bacillus cereus* subgroup A, and designated as *Bacillus cereus* H62L-1.

The partial genetic sequence is shown below.

GAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGC
AAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGAC
GGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGG
AAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTG
AAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTA
GTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGA
GGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAG
GCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC
CGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAG
AACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAA

At the same time a voucher for the sequencing has been submitted to the editor as a supplement.

3.4. Release of flaxseed lignans from the defatted flaxseed flour (DFF)

The newly isolated strain *Bacillus cereus* H62L-1 could release CAG, FAG, and SDG. However, its conversion of SDG to SECO was limited (Fig. 4). The literature indicates that such biotransformations can be attributed to the action of glucuronidases [30], β-glucosidases [31], and demethylases as well [32].

3.5. Growing cells are more tolerant to SDG, while resting cells are more feasible for biotransformation

In Exp. 1, the growing cells produced SDG from DFF at a production rate of 1.08 $mgL^{-1}h^{-1}$ (Table 2), and a peak concentration of 158 mg/L was observed at time of 216 h (Fig. 4). The production of other constituents was comparatively low and reached only 0.12, 0.09, and 0.06 $mgL^{-1}h^{-1}$, for CAG, FAG, and SECO, respectively (Table 2). After reaching its peak level, SDG started to decay at a rate of 0.13 $mgL^{-1}h^{-1}$ to a

concentration of 128 mg/L at h 360 (Fig. 4). In parallel, SECO remained almost unaffected at 2 mg/L throughout the course of the analysis, which indicates a possibly overwhelming reversible reaction (Fig. 4). Instead, in Exp. 2, the growing cells produced SDG from EEF at a transformation rate of 0.12 $mgL^{-1}h^{-1}$. SECO was produced at a very low production rate of 0.01 $mgL^{-1}h^{-1}$ (Table 2). When the culture was changed to resting cells (Exp. 3), the production rates of SDG and SECO increased to 0.15 and 0.12 $mgL^{-1}h^{-1}$, respectively (Table 2), which indicated a better feasibility for using resting cells to produce SECO from EEF (Table 2).

3.6. The optimum concentrations of resting cells and tryptone broth all decreased at 15%

The resting cells at concentrations from 0% to 25%, and tryptone broth from 0% to 75% were tested to determine the optimal concentrations. The optimal concentrations of resting cells and tryptone broth for SDG biotransformation were both 15% (Figs. 5a and 5b; other concentrations not shown here).

3.7. Fermentation with authentic lignans confirmed the consecutive biotransformation reactions

To confirm the consecutive fermentative reactions, authentic lignans were fermented with a concentration of 15% resting cells. The SECO level continuously increased, whereas the SDG level continuously decreased (Fig. 6). Apparently, the slight increase in END was due to a net accumulation of SECO. However, the concentration of END was too low to be further transformed into the next end product, ENL. Hence the level

of ENL steadily declined from the beginning of the analysis, which is consistent with the results of Clavel et al. [4].

3.8. Fermentation with EEA confirmed the consecutive biotransformation reactions

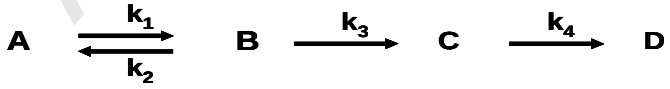
Alternatively the consecutive reaction mode of EEA biotransformation was conducted with 15% resting cells cultured in 15% tryptone broth that contained EEA**.** HPLC analysis showed most of the CAG, FAG and SDG initially present in the EEA (upper spectrum taken at h 0, Fig. 7) was converted to CA, FA, and SECO after fermentation for 24 h (lower spectrum, Fig. 7).

3.9. The substrate-limiting step was during SDG deglucosylation

The kinetic parameters (Table 2) revealed that the conversion of SDG into END and ENL was rather inefficient. The limiting step was apparently the first step — the reversibility in the deglucosylation of SGD to produce SECO. Apparently the equilibrium was reached early at h 48 (Fig. 8).

3.10. Kinetic analysis of the reversible consecutive biotransformation reactions

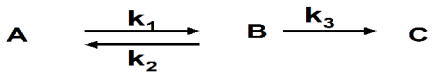
Based on careful inspection of figures 4, 6, 8, and the data listed in Table 2, SDG deglucosylation was found to be a reversible reaction. Assume that the whole sequence of reaction can be expressed as follows (below), and that each step is of first order kinetics with respect to their corresponding substrate.



where A, B, C and D are SDG, SECO, END, and ENL, respectively, and the parameters k_1, k_2, k_3, and k_4, are the rate coefficients for the forward reaction from

SDG (A) to SECO (B), the reverse reaction from SECO to SDG, the forward reaction from SECO (B) to END (C), and the forward reaction from END to ENL (D), respectively.

At the very beginning, soon after the reaction starts, i.e. under the condition that the conversion of A to C into D is still insignificant, the kinetic constants k_4 can be neglected, which actually was the condition observed in tables 2 and 3, and figures 3 and 4. The above consecutive reversible reaction can be simplified to



Based on which the net deglucosylation of A, (SDG) is

$d[A]/dt = -k_1[A] + k_2[B]$ ……..1

The net formation rate of B (SECO) is

$d[B]/dt = k_1[A] - k_2[B] - k_3[B]$ ……..……2

and accordingly, the net formation rate of C (END) is

$d[C]/dt = k_3[B]$ ……..….3

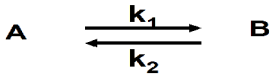
At steady state, Eq. 2 = 0, giving

$k_1[A] = k_2[B] + k_3[B]$ ……...4

Rearrangement of Eq. 4 gives

$k_1/(k_2 + k_3) = [B]/[A]$ …….....5

In a case where the formation rate of END (C) is too small and negligible, these reversible consecutive reactions are further simplified to



which actually was observed in Fig. 4,5 and 8. Hence we have

$k_1/k_2 = [B]/[A]$ ……..6

which in reality has become a conditionally reversible pseudo-first-order reaction. Thus, dada have confirmed the nature of a consecutive reactions starting from SDG to ENL. The data obtained in Fig. 5 and 8 were calculated to produce the relevant kinetic parameters for the description of the reversible consecutive biotransformation reactions, as shown in Eq. 5 (Table 2).

Other literature has demonstrated that the polymerized SDG can be broken down into individual molecules with the cleavage of glucose moieties in the intestines by β-glucuronidases and β-glucosidases [33], an implication in the synergistic biotransforming capability of the intestinal microflora. Through *Bacillus cereus* H62L-1-mediated biotransformation, the production of the mammalian lignans END and ENL was viewed as almost unachievable due to the highly reversible first step between SDG and SECO, which is actually controlled by a specific β-glucosidase [33]. Our kinetic data revealed that the forward and the reverse rate coefficients were very comparable. Substrate loading with authentic pure SDG exhibited a k_1 value of 0.025 h^{-1} (Fig. 5a, Table 3), which reduced to only 0.001 h^{-1} when fermented with EEA and implicated that interfering factors possibly exist in EEA (Fig.8, Table 3). In contrast, the rate coefficient of the reverse reaction for SECO to SDG was 0.077 h^{-1} when EEA

was used, implicating an overwhelming retardation in the conversion of SDG into SECO. As evidenced, the kinetic rate coefficients for the conversion of SECO into END and the subsequent production of ENL exhibited rates of 0.013 h^{-1} and 0.002 h^{-1}, respectively. Moreover, the value of the parameter $k_1/(k_2+k_3)$ ranged within 0.05-1.32 depending on the nature of substrate utilized (Table 3), mostly not favoring the forward reaction. Apparently the reversibility could not be overcome (Table 3). To increase the yield, strategies may involve i) to increasing the purity and concentration of the SGD substrate; ii) using an allosteric inhibitor to block its allosteric site, which would hinder its catalytic affinity for SECO and inhibit the reversibility of the *Bacillus cereus* H62-L1 β-glucosidase; and iii) by genetically modifying the β-glucosidase. Future work will emerge using these technologies.

4. Conclusion

The amount of major lignans present in the alkaline extract of the defatted flaxseed is (in mg/g) are as follows: secoisolariciresinol diglucoside (SDG, 109.80), *p*-coumaric acid glucoside (CAG, 70.21), ferulic acid glucoside (FAG, 68.81), secoisolariciresinol (SECO, 0.49), coumaric acid (Ca, 0.37), and ferulic acid (FA, 0.85). The yield from the biotransformation of SDG to SECO is extremely limited due to the reversibility of the *Bacillus cereus* H62-L1 β-glucosidase which governs the biotransformation of SDG to SECO. The parameters affecting the yield include the purity of the SDG,. and the reversibility of β-glucosidase. To increase the yield of mammalian lignans, we propose i) using high purity SDG substrate at higher concentrations; or ii) using an

allosteric inhibitor to delay the reverse reaction from SECO to SDG; or iii) genetically modifying *Bacillus cereus* strain H62-L1.

Acknowlegements

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Conflict of Interest and Disclosure Statement

The authors do not have any conflict of interest, and the authors have already disclosed any actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence (bias) their work and related potential conflicts of interest including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. Potential conflicts of interest should be disclosed at the earliest possible stage.

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TABLE CAPTION

Table 1. Table 1. HPLC analysis of lignans present in the ethanolic extract from the alkaline hydrolysate of the defatted flaxseed flour before fermentation.[a]

Table 2. The complicate lignan production kinetics for different substrates fermented by different phase of cells under different cultivation conditions[a]

Table 3. Varying kinetic rates revealing the limited productivity of mammalian lignans due to the reversibility of the step from SDG to ENL

Table 1. HPLC analysis of lignans present in the ethanolic extract from the alkaline hydrolysate of the defatted flaxseed flour before fermentation.[a]

	CAG	FAG	SDG	CA	FA	SECO	END	EBL
RT (min)	14.832	16.895 .	21.929	24.696	26.138	30.108	33.782	40.147
Content (mg/g)	70.21 ±4.53	68.81 ±4.30	109.80 ±5.78	0.37 ±0.01	0.85 ±0.03	0.49 ±0.05	NF	NF

[a]RT: retention time. NF: not found

CAG: coumaric acid glucoside. FAG: ferulic acid glucoside. SDG: secoisolariciresinol diglycoside. CA: coumaric acid. FA: ferulic acid. SECO: secoisolariciresinol. Data expressed in mean ±SD (n =6). END and ENL were absent before microbial transformation. The retention of END and ENL were obtained by spiking the authentic END and ENL in to the sample solution.

Table 2. The complicate lignan production kinetics for different substrates fermented by different phase of cells under different cultivation conditions[a]

Experiment/ substrate/culture type	Kinetic data for production rate (mg/L-h)					
	CAG	FAG	SDG	CA	FA	SECO
Exp 1/DFF	-	-	-	-	-	-
/growing cells	-	-	-	-	-	-
Production rate	0.12	0.09	1.08	-	-	0.06
Decay rate post peak point	0.00	0.00	0.13	-	-	0.01
Exp 2/EEF	-	-	-	-	-	-
/growing cells	-	-	-	-	-	-
Production rate	0.09	0.04	0.12	0.00	0.00	0.01
Decay rate post peak	0.00	0.00	0.00	0.00	0.00	0.00
Exp 3/EEF	-	-	-	-	-	-
/resting cells	-	-	-	-	-	-
Production rate	0.10	0.02	0.15	0.00	0.01	0.12
Decay rate post peak point	0.00	0.00	0.00	0.00	0.00	0.02

[a]Exp 1: Growing cells + DFF, fermented for 360 h. Exp 2: Growing cell + EEF fermented for 72 h. Exp 3: Resting cell + EEF, fermented for 72 h. All cultures were incubated at 37^{o}C while striring at 150 rpm. Samplings were performed every 12 h.

Table 3. Varying kinetic rates revealing the limited productivity of mammalian

lignans due to the reversibility of the step from SDG to ENL

Substrate	Biotransformation rate, μg/L-h				
	Reaction step 1	Reaction step 2	Reaction step 3	Reaction step 4	-
SDG[a]	825[a]	196[d]	283[b]	33[b]	-
SDG[b]	629[b]	179[d]	283[b]	33[b]	-
SDG[c]	145[c]	89[d]	283[b]	33[b]	
SECO[b]	450[b]	179[b]	283[b]	33[b]	-
SECO[c]	56[c]	89[d]	283[b]	33[b]	-
END[b]	-	-	283[b]	33[b]	-
ENL[b]	-	-	-	33[b]	-
Substrate	Kinetic parameter, h^{-1}				
	k_1	k_2	k_3	k_4	$k_1/(k_2 + k_3)$
SDG[a]	0.025[a]	0.006[c]	0.013[b]	0.002[b]	1.32
SDG[b]	0.027[b]	0.008[c]	0.013[b]	0.002[b]	1.19
SDG[c]	0.001[c]	0.006[c]	0.013[b]	0.002[b]	0.05
SECO[b]	0.002[b]	0.007[c]	0.013[b]	0.002[b]	0.10
SECO[c]	0.028[c]	0.077[c]	0.013[b]	0.002[b]	0.31
END[b]	-	-	0.013[b]	0.002[b]	-
ENL[b]	-	-	-	0.002[b]	-

[a]Calculated from Fig. 5a: substrate pure authentic SDG (initial concentration 32.8 mg/L). [b]Calculated from Fig. 6. Substrate: authentic lignan mixture (each initial concentration was 27 mg/L). [c]Calculated from Fig. 8. Substrate EEA, incubated at 37°C for 72 h with a stirring rate at 150 rpm. The calculation was performed using the first kinetic order equations Eq. 1 and Eq. 3. For transit reaction, the first order kinetic equation $dC/dt = k_1C$ (approximately in form of $\Delta C/\Delta C = k_1C$) was used to calculate the kinetic parameters from the figures revealing linear correlation in each figure.

FIGUTRE LEGEND

Fig. 1. The structure of main polyphenolics present in the desiccated defatted flaxseeds.

Fig. 2. The microbial biotransformation pathway from SDG to END and ENL (modified from Clavel et al, 2007).

Fig. 3. HPLC analysis of lignans present in the ethanolic extract of the alkaline hydrolysate of the defatted flaxseed flour (EEA) before fermentation. The EEA was filtered with 0.45μ Micropore filter. An aliquot 20 μL of the filtrate was subjected to HPLC analysis. The retention time (min) was: coumaric acid glucoside (CAG, 14.832). Ferulic acid glucoside (FAG, 16.895). Secoisolariciresinol diglucoside (SDG, 21.929). Coumaric acid (CA, 24.696). Ferulic acid (FA, 26.138). Secoisolariciresinol (SECO, 30.108). END and ENL were absent in the original sample. Their retentions time were obtained by spiking with authentic END and ENL. The retention times were33.782 min for enterodiol (END) and 40.147 min for enterolactone (ENL), respectively.

Fig. 4. Preliminary test on the release of lignans from the defatted flaxseed flours (DFF) by *Bacillus cereus* H62L-1.

To 2 mL of the DFF polymer solution, 8 mL tryptone broth and 0.5 mL of fresh growing culture were added and incubated at 37 ^{o}C for 360 h. The fermentation fluid was filtered with 0.45μ Micropore filter. An aliquot 20 μL of the filtrate was subjected to HPLC analysis.

Fig. 5. Effect of the resting cell- (5a) and the tryptone broth (5b) concentrations on the biotransformation of the authentic secoisolaricirecinol (SDG) by *Bacillus cereus H62L-1* resting cells.

Fig. 6. Higher biotransformation rate by *Bacillus cereus* H62L-1 resting cells found for the authentic lignans.

The initial concentration of authentic SDG, SECO, END, and ENL was 27 mg/L each. The authentic lignans were respectively fermented at 37^{o}C with 20% resting cells at a stirring speed 150 rpm for 36 h. The fermentation fluid was filtered with 0.45μ Micropore filter. An aliquot 20 μL of the filtrate was subjected to HPLC analysis.

Fig. 7. Sequential appearance of CA, FA, and SECO through deglucosylation of CAG, FAG, and SDG by *Bacillus cereus* H62L-1. Substrate, EEA. (Upper: before fermentation. Lower: after fermented for 12, 24, and 36 h, respectively). To 2 mL of the EAA polymer solution, 8 mL tryptone broth and 0.5 mL of fresh growing culture were added and incubated at 37 ^{o}C for 36 h. The fermentation fluid was filtered with 0.45μ Micropore filter. An aliquot 20 μL of the filtrate was subjected to HPLC analysis.

Fig. 8. Apparent reversibility found in the deglucosylation step for biotransforming secoisolaricirecinol (SDG) into secoisolariciresinol (SECO).

Fermentation condition: substrate EEA in 10 mL reaction mixture containing tryptone broth 15%, temperature, 37^{o}C; stirring speed, 150 rpm; time period, 72 h. The fermentation fluid was filtered with 0.45μ Micropore filter. An aliquot 20 μL of the filtrate was subjected to HPLC analysis.

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Figure 1

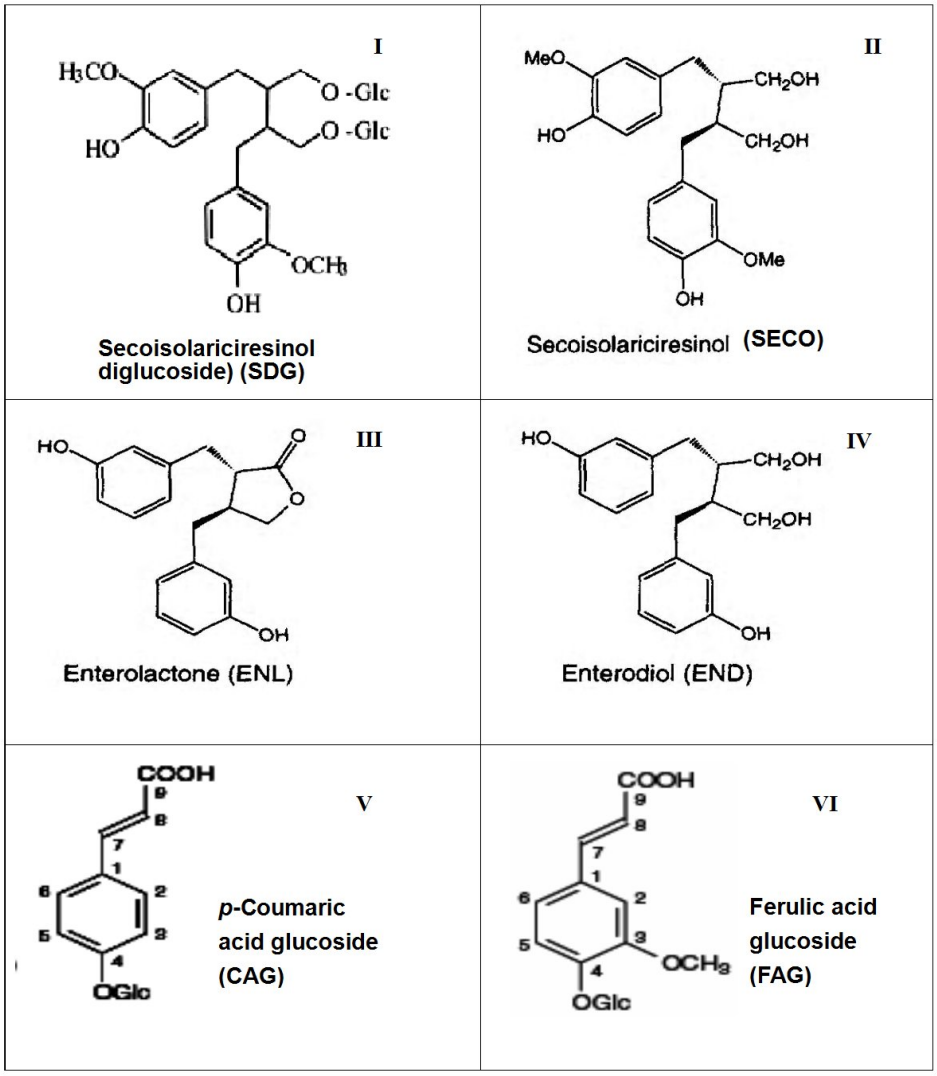

Figure 2

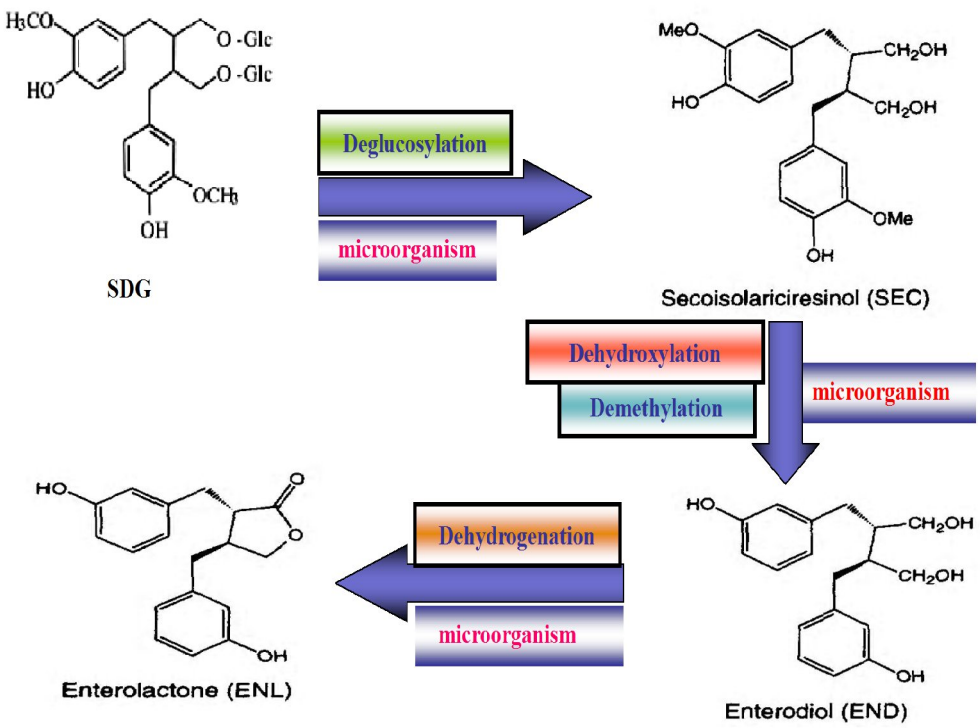

Figure 3 HPLC Chromatogram

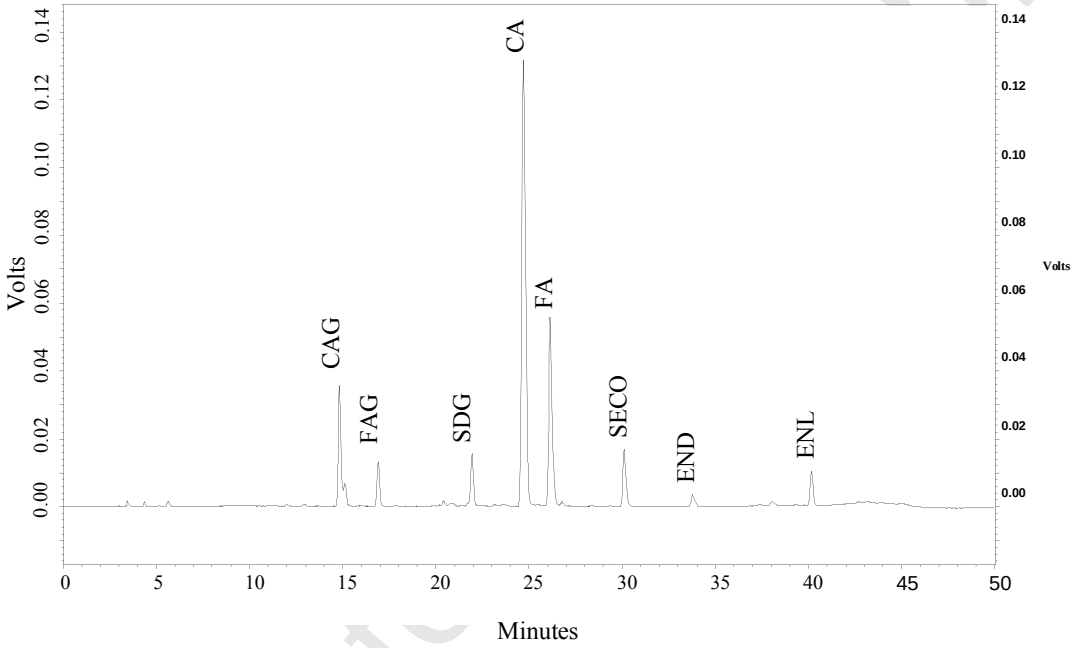

Figure 4

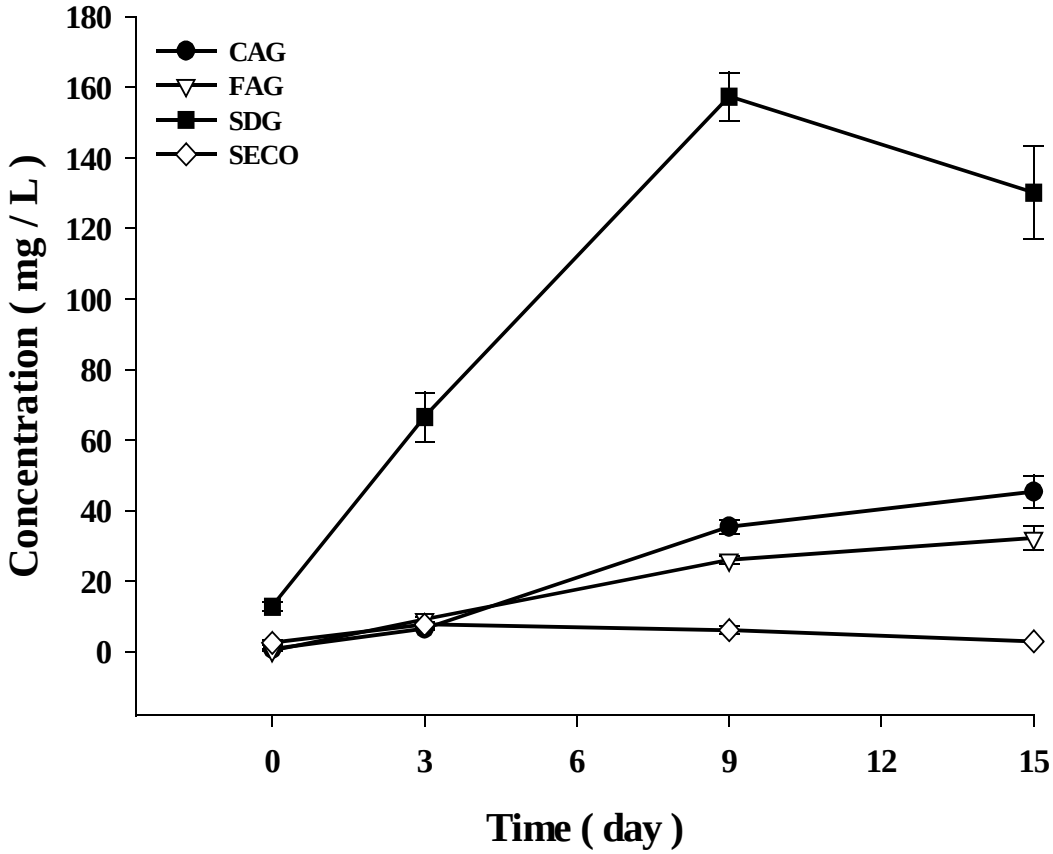

Figure 5
Figure 5a

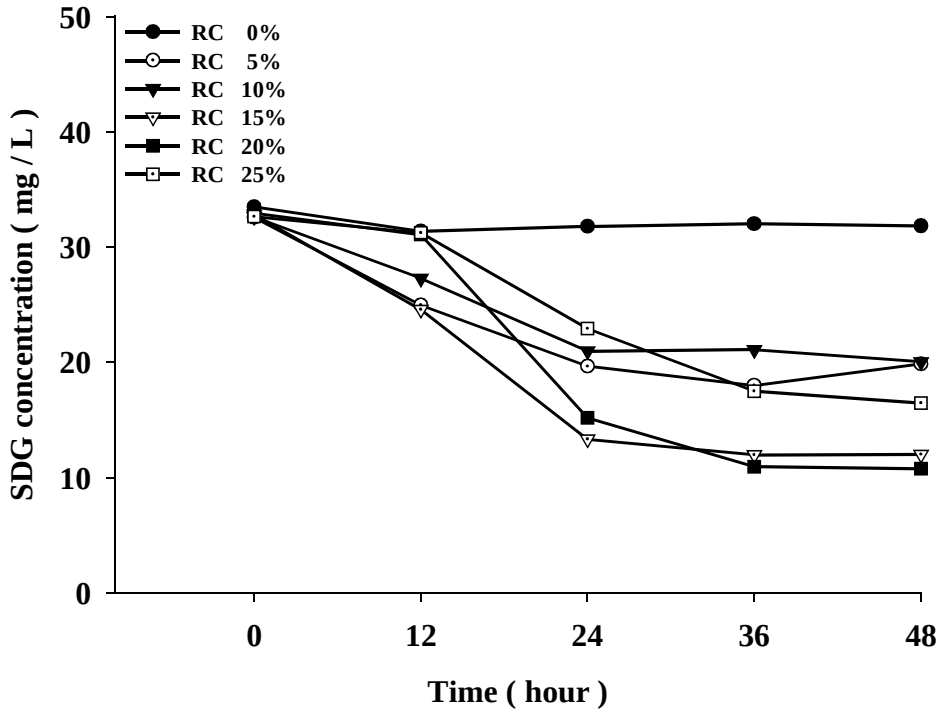

Figure 5b

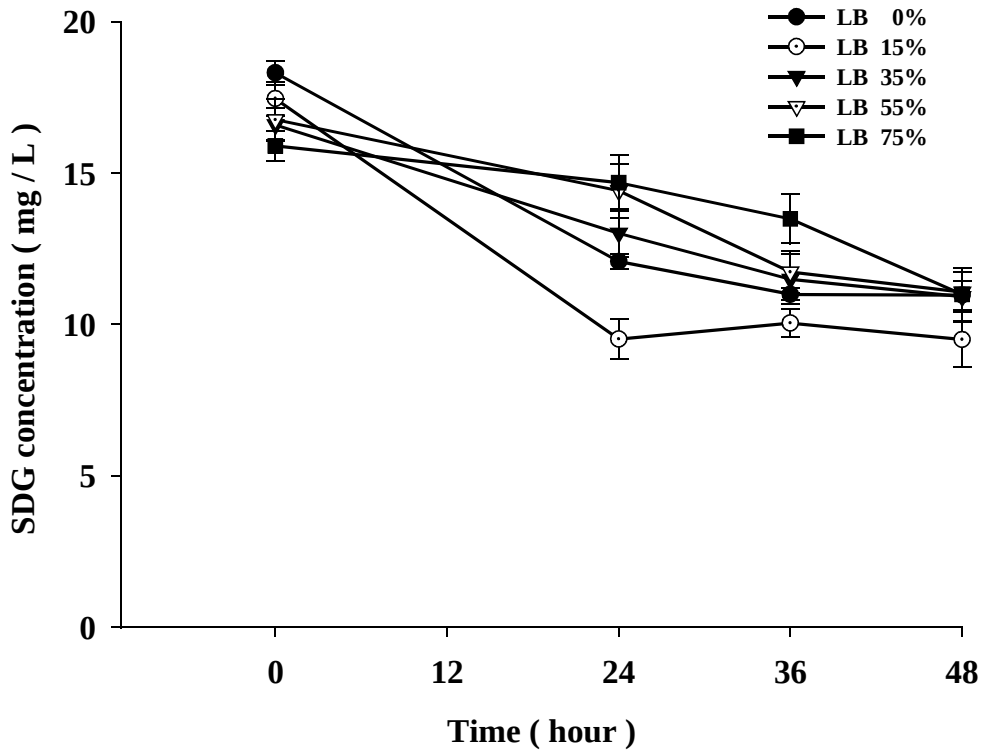

Figure 6

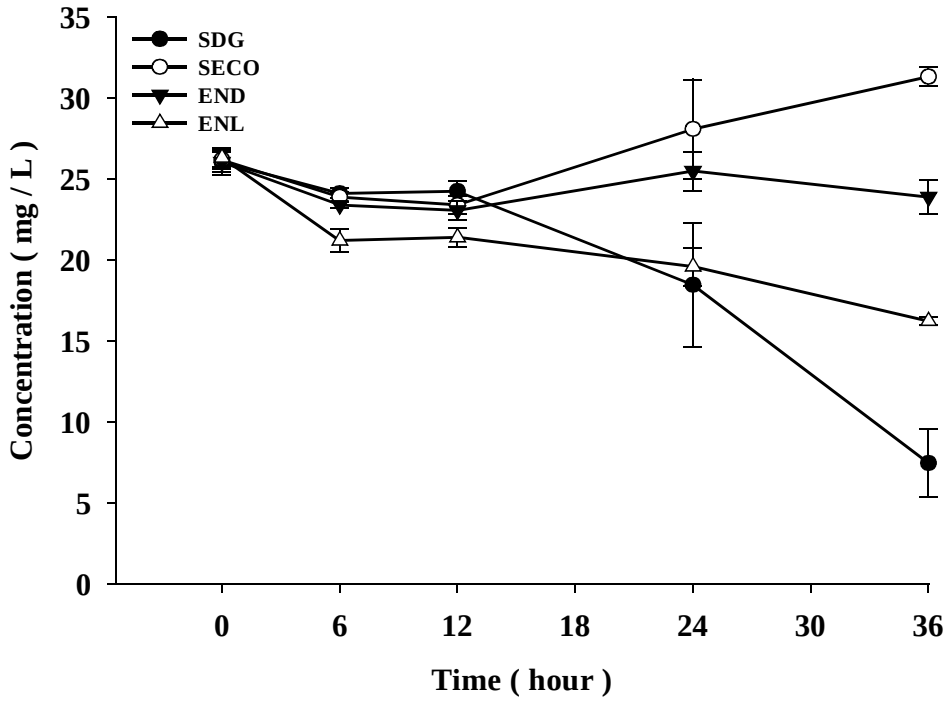

Figure 7

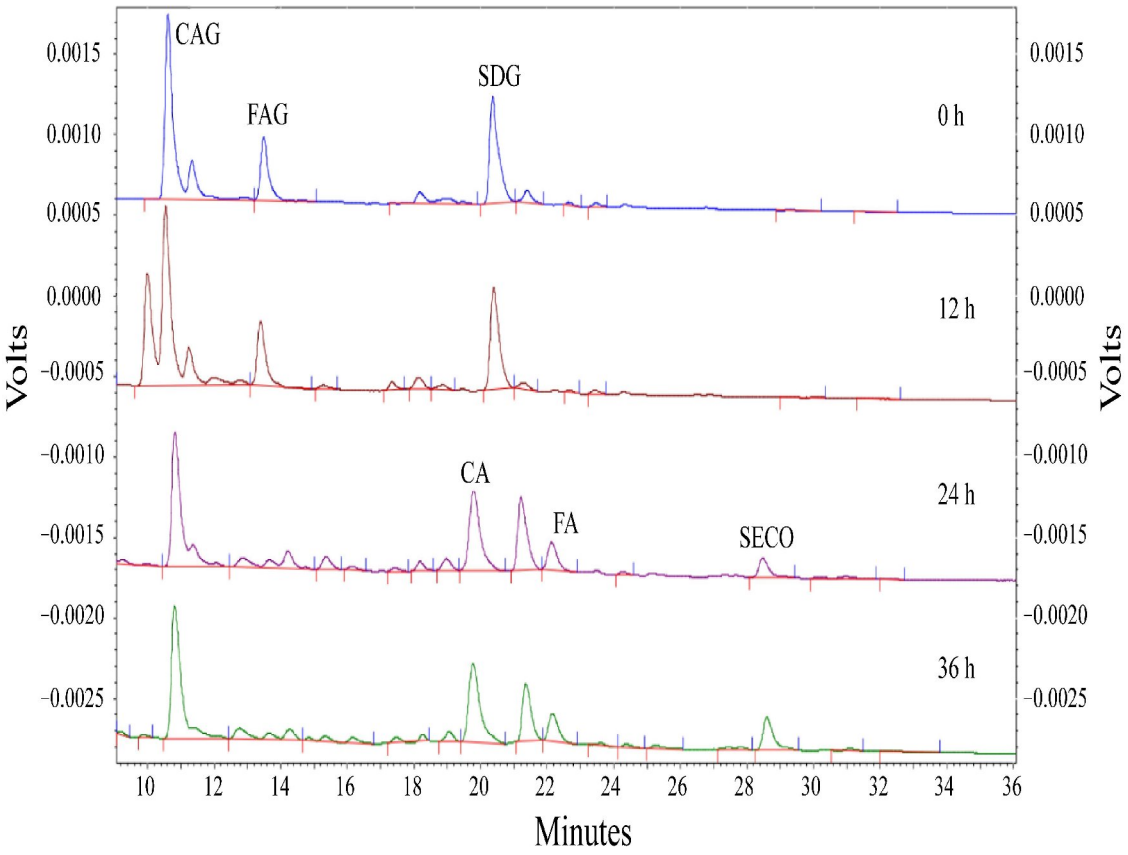

Figure 8