Biotransformation of soybean saponin to soyasapogenol B by *Aspergillus parasiticus*

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Objectives
The aim of this study was to select of the most potent fungus that is able to hydrolyze soybean saponin (SS) to soyasapogenol B (SB). The selected fungus was cultivated under different physiological conditions to evaluate its ability to transform SS to achieve the maximal conversion output.

Materials and methods
Within 72 h, the biotransformation of SS by *Aspergillus parasiticus*, followed by isolation and purification of SB as a main product were carried out. The identity of SB was established by determination of its RF value and IR, mass spectra, and NMR spectra. Furthermore, different sets of experiments were carried out to enhance the activity of the tested organism and consequently, SB production.

Results and conclusion
Screening of different fungal isolates for transformation of SS to SB revealed that *A. parasiticus* produced the highest yield of SB. The maximum SB yield was obtained using a production medium composed of (%, w/v): malt extract, 4; yeast extract, 2; KH_2PO_4, 0.2; $(NH_4)_2SO_4$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.03; $CaCl_2 \cdot 2H_2O$, 0.03; galactose, 0.5; and SS, 3 (pH 8). The medium was inoculated with 6% (v/v) inoculum of a 72 h old culture and incubated on a rotary shaker (150 rpm) at 30°C for 72 h. Under these optimal conditions, the cell biotransformation efficiency was increased from 13.44 to 65%.

Keywords:
Aspergillus parasiticus, biotransformation, soyasapogenol B, soybean saponin

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Introduction

Saponins are structurally diverse molecules that are chemically referred to as triterpenes and steroid glycosides. They consist of nonpolar aglycones coupled with one or more monosaccharide moieties [1]. This combination of polar and nonpolar structural elements in their molecules explains their soap-like behavior in aqueous solutions.

Soyasaponins are a group of oleanane triterpenoids found in soy and other legumes. They are divided into three groups, based on the structure of the aglycone moiety, the A, B, and E saponins [2]. Soyasapogenols A, B, and E are conjugated as glycosides in soy [3,4]. The current consensus is that soyasapogenols A, B, and E are true aglycons, whereas soyasapogenols C, D, and E are artifacts of hydrolysis that occur during the isolation process of A, B, and E soyasapogenols .

Soyasaponins have various physiological effects including hepatoprotective [5], anticarcinogenic [6], antiviral [7], and anti-inflammatory [8] activities. Soyasapogenol B (SB), obtained from soybean saponin (SS), is known to have hepatoprotective [9], antiviral [10], antimutagenic [11], anti-inflammatory [8], and growth suppressing effects on cells derived from human colon and ovarian cancer [11,12].

Results from in-vitro fermentation suggest that colonic microflora readily hydrolyzed SS to aglycones [2]. These observations suggested that the dietary chemopreventive effects of SS against colon cancer may involve alteration by the microflora [12]. There is some evidence, as with many other saponins, that bioactivity of SS is increased as sugar moieties are eliminated from the saponin structure, thereby reducing the polarity.

Aglycones, soyasapogenols, are produced by acid hydrolysis of saponins, but there have been reports of aglycone production by microorganisms. Kudou *et al.* [13] cultured 158 strains of the genus *Aspergillus* in a medium containing SS and reported that 26 of them had a marked SS hydrolase activity. Watanabe *et al.* [14] isolated a SS hydrolase from *Neocosmospora vasinfecta var. vasinfecta* PF1225, a filamentous fungus that can degrade SS and generate SB. Recently, Amin and Mohamed [15] reported the production of SB (86.3%) from SS using immobilized *Aspergillus terreus* on a loofah sponge. The aim of this study was to select the most potent fungus that is able to hydrolyze SS to SB. The selected isolate was cultivated under different physiological conditions to evaluate its ability to transform SS to achieve the maximal conversion output.

Materials and methods

Cultivation of fungal isolates

The different fungal isolates used in this work (Table 1) were donated by the Center of Cultures of Chemistry of

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Table 1 Bioconversion of soybean saponin to soyasapogenol B by different fungal strains

	Soyasapogenol B	
Fungal isolates	mg/100 ml	Molar yield (%)
Aspergillus fumigatus	6.8	2.85
Aspergillus flavus	22.34	9.38
Aspergillus niger	18.58	7.8
Aspergillus parasiticus	32	13.44
Aspergillus ruber	4.92	2.06
Rhizopus riori	14.6	6.13
Penicillium aurantiacum	–	–
Penicillium waksmanii	1.86	0.78
Penicillium frequentans	9.6	4.03
Penicillium cyclopium	8.12	3.41
Trichoderma harzianum	3.9	1.63
Trichoderma viride	3.82	1.6

Strains were cultivated on a transformation culture medium consisting of (%, w/v): malt extract, 4; yeast extract, 2; KH_2PO_4, 0.2; $(NH_4)_2SO_4$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.03; $CaCl_2 \cdot 2H_2O$, 0.03; and SS, 1 (pH 5.7) at 150 rpm and 30 ± 2°C for 72 h.
SS, soybean saponin

Natural and Microbial Products Department, National Research Center (Cairo, Egypt). They were maintained on potato dextrose agar slants at 4°C and subcultured at intervals of 1–2 months. Unless otherwise stated, the fermentations were carried out in 250 ml Erlenmeyer flasks containing 100 ml of the fermentation medium composed of (%, w/v): malt extract, 4; yeast extract, 2; KH_2PO_4, 0.2; $(NH_4)_2SO_4$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.03; $CaCl_2 \cdot 2H_2O$, 0.030; and SS, 1 (pH 5.7) [16]. The flasks were inoculated with 6% inoculum and agitated on a rotary shaker at 150 rpm at 30 ± 2°C for 72 h.

General assessment of the chemicals and instruments used

SS (50%) was purchased from Organic Technologies Co. (Coshocton, Ohio, USA). Potato dextrose agar and yeast extract were products of Biolife Italiana (Milano, Italy). Bacto malt extract and bacto peptone were purchased from Difco Laboratories (New Jersey, USA). ^{1}H NMR and ^{13}C NMR spectra were measured using a Bruker AMX 500 instrument (Weizmann Institute of Science Chemical, Rehovot, Israel) operating at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR. Samples were dissolved in fully deuterated dimethyl sulfoxide (DMSO-d_5). The chemical shifts (δ) are reported in ppm and the coupling constants (J) in Hz. Mass spectra were measured using a Finnigan mat. SSQ 7000 instrument at an ionization voltage of 70 eV and EI mode.

Quantitative analysis of soyasapogenol B

At the end of the biotransformation period, the reaction mixture was extracted twice with double the volume of ethyl acetate. Thereafter, the organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue was dissolved in a chloroform–methanol mixture (1 : 1) and mounted on thin-layer chromatography (TLC) plates. The plate was first chromatographed for soyasapogenols using the above-mentioned solvent system and then for SS using a solvent system comprising chloroform–methanol–acetic acid (10 : 20 : 1, v/v). SS and SB were detected on TLC plates by spraying with 10% H_2SO_4 and then heating for 10 min at 110°C; they were then quantitatively analyzed using a TLC-scanner (Shimadzu CS-9000 dual wavelength flying spot, thin layer chromato-scanner, Tokyo, Japan) at λ equal to 530 nm [16]. The obtained weight of SB was calculated by calibration of the line obtained from the standard sample using the area under the curve for the biotransformation products in each chromatogram.

$$\text{SB molar yield (\%)} = \frac{\text{Weight of soyasapogenol B/MW of soyasapogenol B}}{\text{Weight of soyasaponin I/MW of soyasaponin I}} \times 100,$$

where MW is the molecular weight and soyasaponin I represents SS.

Separation and identification of the biotransformation products

After cultivation of *Aspergillus parasiticus* on the biotransformation culture medium containing 1% SS, the resulting filtrate (500 ml) was extracted twice with ethyl acetate, and the organic layer was concentrated under reduced pressure to obtain an oily sample (415 mg). A preparative silica gel plate (silica gel 60 F-254 aluminum plates; Merck, Darmstadt, Germany) was spotted and developed using the same solvent system (benzene : ethyl acetate : acetic acid; 24 : 8 : 1, v/v). The areas containing soyasapogenols were detected by a slight discoloration on the plates, and these sections were scraped, extracted with chloroform : methanol (1 : 1), and evaporated to dryness. This led to isolation of compound I (56 mg) as the main product.

Compound I

Compound I was identified as SB, with a melting point of 230°C. The H^1 NMR (DMSO-d_5) results were as follows: δ at 5.18 (t, 1H, $J_{12,11\alpha} = J_{12,11\beta} = 4$ Hz, H-12), 4.85 (d, 1H, $J_{24a\text{-}24b} = 4.6$ Hz, H-24a), 4.14 (dd, 2H, H-3, and H-21), 4.05 (d, 1H, $J_{24b\text{-}24a} = 4.6$ Hz, H-24b), 3.82 (d, 1H, $J_{22\alpha\text{-}21\beta} = 8.4$ Hz, $J_{22\alpha,21\alpha} = 2.4$ Hz, H-22α), 1.2 (s, 3H, H-23), 1.18 (s, 3H, H-27), 0.95 (s, 3H, H-28), 0.90 (s, 3H, H-26), 0.84 (s, 6H, H-25), 0.82 (s, 3H, H-29), and 0.80 (s, 3H, 30) and the ^{13}C NMR (CD_3Cl) results were shown in Table 2.

Optimization of soybean saponin biotransformation using *Aspergillus parasiticus*

Optimization of the environmental conditions for microbial biotransformation processes on a laboratory scale is important to obtain information for the scaled-up production of the target product in a large-scale fermentor. The parameters assessed were pH (4, 5, 5.7, 6, 7, 8, and 9) of the medium, inoculum size (1, 2, 3, 4, 5, 6, 8, and 10%, v/v) and age (24, 48, 72, and 96 h), duration of the biotransformation process (24, 48, 72, 96, 120, and 144 h), SS concentration (0.5, 1, 2, 3, and 4%, w/v), incubation temperature (20, 25, 30, 35, and 40°C), and shaking incubator speed (static,100, 150, 200, and 250 rpm). For examining the effect of the cultivation medium composition on the biotransformation process, different levels of either malt extract (2, 3, 4, 5, and 6%,

Table 2 ^{13}C NMR assignments of soyasapogenol B

Carbon number	Soyasapogenol B
1	38.13
2	27.14
3	78.57
4	42.05
5	55.34
6	18.54
7	32.77
8	41.14
9	47.11
10	36.24
11	23.13
12	121.46
13	144.03
14	41.56
15	25.45
16	27.83
17	36.87
18	44.51
19	46.01
20	30.11
21	41.56
22	73.98
23	22.80
24	62.94
25	15.57
26	16.50
27	24.99
28	28.23
29	32.59
30	20.23

w/v) or yeast extract (0.5, 1, 2, 2.5, and 3%, w/v), different carbon sources (glucose, galactose, mannose, sucrose, arabinose, and starch), and different concentrations of galactose (0.5, 1, 2, 3, 4, and 5% w/v) were individually used.

Results and discussion

Screening experiments

Twelve fungal isolates were screened for their saponin-hydrolyzing abilities to produce SB from the SS that was added to the culture medium. Results in Table 1 indicate different capacities of the tested cultures to produce SB. *P. aurantiacum* failed to perform the desired reaction, whereas the other fungal isolates (*Aspergillus flavus*, *Aspergillus fumigates*, *Aspergillus niger*, *Aspergillus parasiticus*, *Aspergillus ruber*, *Penicillium cyclopium*, *Penicillium frequentans*, *Penicillium waksmannii*, *Rhizopus riori*, *Trichoderma harzianum*, and *Trichoderma viride*) could. Among the 12 examined fungal cultures, *A. parasiticus* produced the highest yield of SB; it could transform about 13.44% of the added SS, with the formation of 32 mg/100 ml SB. In this connection, Kudou *et al*. [13] reported that 26 of 158 strains of the genus *Aspergillus* had a marked SS hydrolase activity when cultured in a medium containing SS. Moreover, Watanabe *et al*. [17] purified a SS hydrolase from *Aspergillus oryzae* PF1224.

Identification of the biotransformation products

As *A. parasiticus* was cultivated for 72 h on a medium containing 1% SS; compound I was isolated as a major product (about 80%) in addition to some other minor by-products. Physicochemical characteristics and various spectral data of the obtained compound I were identical to those of standard SB. Compound I produced red color with sulfuric acid alone or with Liebermann–Burchard reagent for the triterpenes. The molecular formula was assigned to be $C_{30}H_{50}O_3$ from the EI-mass spectra (458 *m/z*). The presence of seven tertiary methyl singlets (δ 0.8–1.2) and a triplet olefinic proton at δ 5.18 (t, 1H, $J_{12,11\alpha} = J_{12,11\beta} = 5$ Hz, H-12) in the NMR spectra suggested a olean-12-en structure with three hydroxyl groups. The hydroxyl groups were identified as being attached at C-3, C-22, and C-24 from the H^1 and ^{13}C NMR spectral data. The downfield shift of both C-3 and C-22 (δ 78.57 and 73.98, respectively) in the ^{13}C NMR spectrum suggested that two hydroxyl groups were attached at these positions. The third hydroxyl group was supported at C-24 by the presence of two signals at δ 4.85 (d, 1H, $J_{24a\text{-}24b} = 4.6$ Hz, H-24a) and δ (d, 1H, $J_{24b\text{-}24a} = 4.6$ Hz, H-24b), in addition to a methylene carbon signal at 62.94 ppm in the ^{13}C NMR spectrum. The signal at δ 3.82 (d, 1H, $J_{22\alpha\text{-}21\beta} = 8.4$ Hz, $J_{22\alpha,21\alpha} = 2.4$ Hz, H-22α) was assigned as the H-22α proton, which suggested a β-orientation of the oxygen atom. Therefore, compound I was identified as 3 β, 22 β, 24-trihydroxyolean-12 (13)-ene (SB). All spectral data were in agreement with those published by Kitagawa and colleagues [18,19].

Optimization of soybean saponin biotransformation by *Aspergillu sparasiticus*

Effect of pH

Results presented in Table 3 show that the highest SS conversion activities were maintained within the pH range of 7–9; however, the biotransformation process was markedly impedd at pH values below 5.7. In addition, the initial pH values of the medium (4–9) were found to be shifted toward more acidic values (3.39–6.83) at the end of the bioconversion process. A maximum concentration of SB (89.39 mg/100 ml) corresponding to a molar yield of 37.59% was obtained at pH 8. These findings supported the data reported by Amin *et al*. [19] for the bioconversion of SS to SB by *A. terreus*. Kudou *et al*. [20] found that saponin hydrolase enzyme from *A. oryzae* KO-2 was stable at pH values ranging from 5.0 to 8.0.

Effect of inoculum size

Results illustrated in Fig. 1 indicate that the yield of SB was positively correlated to the increase in the inoculum size up to 6% inoculum (v/v), corresponding to 0.0568 mg cell dry weight, which led to the highest yield of SB (37.59%). In contrast, an increase or decrease in the inoculum size led to a gradual decrease in the SB yield.

Effect of the incubation period

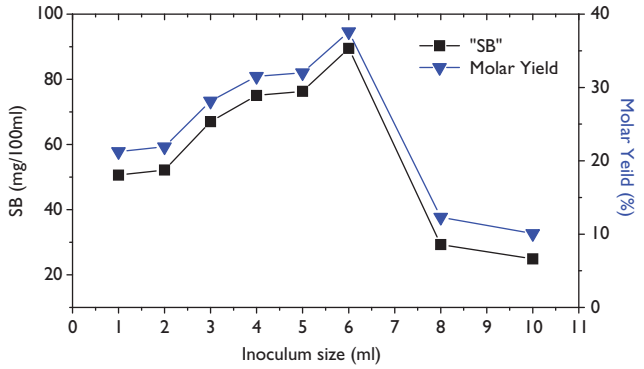
The capacity of *A. parasiticus* to transform SS proved to be markedly affected by the duration of the transformation process. As shown in Fig. 2, biotransformation of SS to SB increased gradually with increase of the incubation period until the maximum value of 37.59% after 72 h was reached, giving an SB yield of 89.5 mg/100 ml. However, this yield sharply decreased upon increasing the time more than 72 h, probably due to a further metabolism of

Table 3 Effect of different initial pH values on production of soyasapogenol B from soybean saponin by *Aspergillus parasiticus*

		Soyasapogenol B	
Initial pH	Final pH	mg/100 ml	Molar yield (%)
5	4.10	11.38	4.78
5.7	4.96	32	13.44
6	5.03	64.33	27.01
7	5.71	83.16	34.92
8	7.22	89.5	37.59
9	6.83	83.39	35.02

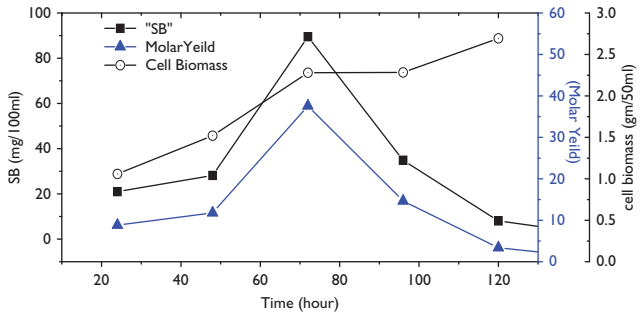
Initial medium pH was adjusted using 1N HCl and 1N KOH at different pH values. *Aspergillus parasiticus* was cultivated on a transformation culture medium at 150 rpm and 30 ± 2°C for 72 h.

Figure 1



Effect of inoculum size on production of soyasapogenol B (SB) from soybean saponin by *Aspergillus parasiticus.* Biotransformation was performed on a transformation culture medium (pH 8) inoculated separately with different inoculum sizes. Flasks were incubated at 150 rpm and 30 ± 2°C for 72 h.

Figure 2



Duration of soyasapogenol B (SB) accumulation during hydrolysis of soybean saponin by *Aspergillus parasiticus. A. parasiticus* was cultivated on a transformation culture medium at pH 8, 150 rpm, and 30 ± 2°C. Molar yield of soyasapogenol B and cell dry weight were determined at different time intervals.

the product. Watanabe *et al.* [14] isolated a SS hydrolase from *Neocosmospora vasinfecta var. vasinfecta* PF1225 after a 72 h incubation period. Moreover, the cell biomass yields were determined at different time intervals (24, 48, 72, 96, 120, and 144 h) and were found to be 2.118, 3.04, 4.558, 4.566, and 5.386 g/100, respectively. Therefore, the trends of SB production and cell growth were roughly equivalent.

Effect of the culture medium composition

Results given in Figs 3 and 4 indicate that *A. Parasiticus* acts optimally at malt extract concentrations of 40 g/l and yeast extract concentrations of 20 g/l, producing an SB yield of 37.59%. Lower or higher levels of malt or yeast extract gave lower yields of SB. Watanabe *et al.* [14] used the same concentrations of malt and yeast extracts to isolate a SS hydrolase from *Neocosmospora vasinfecta var. vasinfecta* PF1225.

As regards the additional carbon sources, results illustrated in Fig. 5 clearly indicate that the maximum yield of SB (41.6%) was achieved when galactose was added to the transformation medium; this is may be due to the enhanced growth of the fungus by using lactose as the carbon source. In contrast, the other tested carbon sources supported comparatively lower conversion estimates and were thus excluded.

Moreover, the effect of different levels of galactose on SB production was studied. Data given in Fig. 6 reveal that a low level of galactose (0.5%) supported maximum SB production (49%), whereas increasing galactose levels over 1% resulted in a dramatic decrease in SB production, possibly because the cells preferred the easily oxidizable galactose as an exclusive carbon source and repressed the induction of saponin-hydrolyzing activity [19].

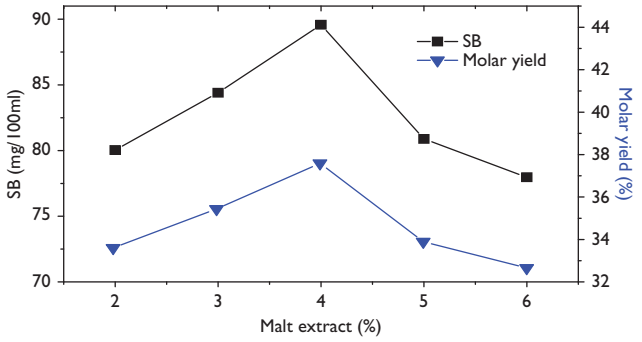
Effect of soybean saponin levels

Kudou *et al.* [20] reported that saponin hydrolase was an enzyme induced by the existence of SS as it has high substrate specificity for the glucuronide bonds of glycosides. Thus, to enhance the productivity, different substrate (SS) concentrations ranging from 0.5 to 4% (w/v) were supplemented to the transformation culture medium at the inoculation time. Results given in Fig. 7 indicate that molar yields of SB increased on increasing the amounts of SS supplemented to the culture medium up to the 3% level. Above the latter concentration, the yield of SB decreased gradually; this is may be due to inhibition of the SS hydrolase on increasing the substrate concentration to more than 3%. Kudou *et al.* [20] indicated that SS hydrolase from *A. oryzae* KO-2 is inhibited by increasing the substrate level above the optimum concentration (2.5 mmol/l).

Effect of incubation temperature

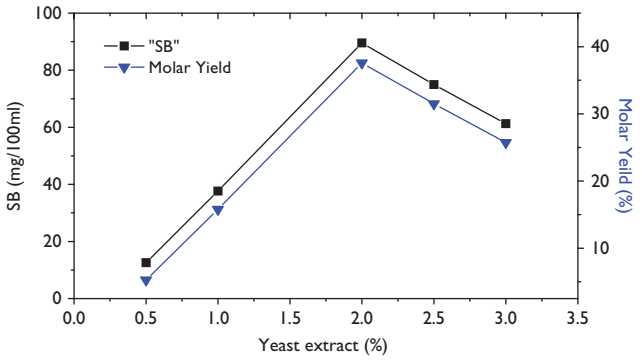
Results in Fig. 8 show that relatively high SB yields were maintained at temperatures ranging from 25 to 35°C. Maximum SS conversion (65%) was achieved at 30°C, leading to a production of 464.24 mg/100 ml SB. Watanabe *et al.* [14] cultivated *Neocosmospora vasinfecta var. vasinfecta* PF1225 on an MY medium at 25°C to isolate a SS hydrolase; this means that the optimal incubation temperature depends on the type of organism used.

Figure 3



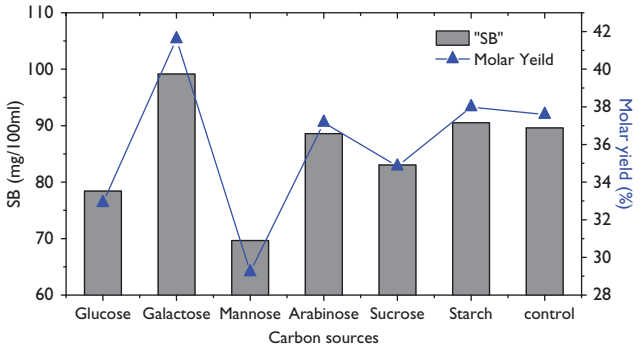
Effect of malt extract concentration on production of soyasapogenol B (SB) from soybean saponin by *Aspergillus parasiticus*. *A. parasiticus* was cultivated on a transformation culture medium supplemented with varying amounts of malt extract (2–6%, w/v) at pH 8, 150 rpm, and 30 ± 2°C for 72 h. Control treatment: using 4% malt extract.

Figure 4



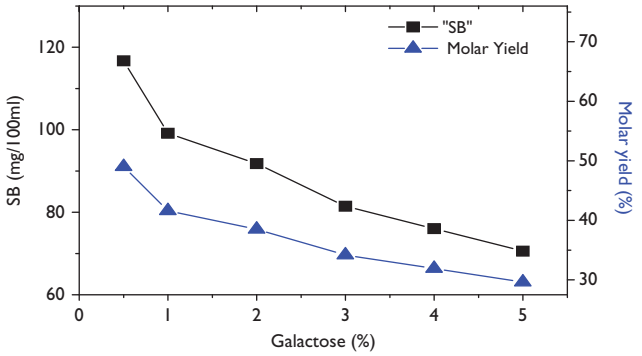
Effect of yeast extract concentration on production of soyasapogenol B (SB) from soybean saponin by *Aspergillus parasiticus*. *A. parasiticus* was cultivated on a transformation culture medium supplemented with varying amounts of yeast extract (0.5–3%,w/v) at pH 8, 150 rpm, and 30 ± 2°C for 72 h. Control treatment: using 2% yeast extract.

Figure 5



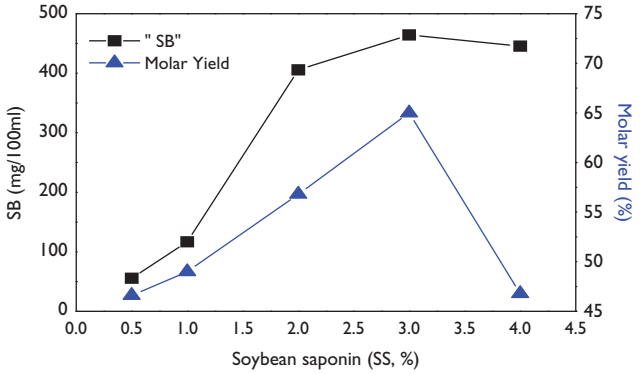
Effect of adding different carbon sources to the fermentation medium on soyasapogenol B (SB) production. *Aspergillus parasiticus* was cultivated on a transformation culture medium supplemented with 1% (w/v) of one of these carbon sources at pH 8, 150 rpm, and 30°C for 72 h. Control treatment: without addition of the carbon source.

Figure 6



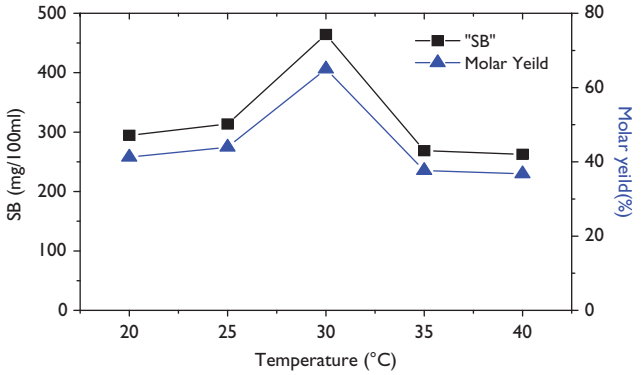
Effect of galactose concentration on soyasapogenol B (SB) production. *Aspergillus parasiticus* was cultivated on a transformation culture medium supplemented with different concentrations of galactose (0.5–5%, w/v) at pH 8, 150 rpm, and 30°C for 72 h. Control treatment: using 1% galactose.

Figure 7



Effect of substrate concentration on production of soyasapogenol B (SB) from soybean saponin (SS) by *Aspergillus parasiticus*. *A. parasiticus* was cultivated on a transformation culture medium supplemented with different levels of SS (0.5–4%, w/v) at pH 8, 150 rpm, and 30 ± 2°C for 72 h. Control treatment: using 1% SS.

Figure 8



Effect of different temperature values on production of soyasapogenol B (SB) from soybean saponin (SS) by *Aspergillus parasiticus*. *A. parasiticus* was cultivated on a transformation culture medium composed of (%, w/v): malt extract 4; yeast extract, 2; KH_2PO_4, 0.2; $(NH_4)_2SO_4$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.03; $CaCl_2 \cdot 2H_2O$, 0.03; and SS, 3 (pH 8). Flasks were incubated at different temperatures and 150 rpm for 72 h.

Conclusion

A. parasiticus was screened and selected on the basis of its ability to hydrolyze SS, producing a high yield of SB. A maximum conversion value of 65% was obtained using a production medium composed of (%, w/v): malt extract, 4; yeast extract, 2; galactose, 0.5; and SS, 3 (pH 8). The medium was inoculated with 6% (v/v) inoculum and incubated at 30°C for 72 h. Under these optimal conditions, the SB molar yield increased from 13.44 to 65%.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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