Biotransformation and recovery of the isoflavones genistein and daidzein from industrial antibiotic fermentations

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Abstract The objective of this study was to follow the metabolic fate of isoflavone glucosides from the soybean meal in a model industrial fermentation to determine if commercially useful isoflavones could be harvested as coproducts from the spent broth at the end of the fermentation. The isoflavone aglycones, genistein, and daidzein together make up 0.1–0.2 % of the soybean meal by weight but serve no known function in the manufacturing process. After feeding genistein to washed cells of the erythromycin-producing organism, *Saccharopolyspora erythraea*, the first biotransformation product (Gbp1) was determined by X-ray crystallography to be genistein-7-O-α-rhamnoside (rhamnosylgenistein). Subsequent feeding of rhamnosylgenistein to growing cells of *Saccharopolyspora erythraea* led to the production of a second biotransformation product, Gbp2. Chromatographic evidence suggested that Gbp2 accumulated in the spent broth of the erythromycin fermentation. When the spent broth was hydrolyzed with acid or industrial enzyme preparations, the isoflavone biotransformation products were returned back to their parental forms, genistein and daidzein, which were then recovered as coproducts. Desirable features of this method are that it does not require modification of the erythromycin manufacturing process or genetic engineering of the producing organism to be put into practice. A preliminary investigation of five additional antibiotic fermentations of industrial importance also found isoflavone coproduct potential.

Keywords Isoflavones · Genistein · Daidzein · Coproduct · Soybean · Fermentation · *Saccharopolyspora erythraea* · 7-O-rhamnosylgenistein

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Introduction

Fermentation manufacturing is a controlled microbiological process that converts agricultural products, such as soybean meal and corn starch, into pharmaceutical products such as antimicrobials, anti-cancer agents, anti-virals, immunosuppressants, herbicides, insecticides, and other natural products (Bérdy 2005). Although fermentation manufacturing of new products can often be improved through modifications of the strain and/or process (Queener and Lively 1986), mature fermentations are more difficult to improve. Therefore, in this study, we investigated a new method for improving the economics of fermentation manufacturing: through the recovery of value-added isoflavone coproducts from the spent fermentation broth. Our principal

model system was the erythromycin fermentation of *Saccharopolyspora erythraea* (Minas 2005).

Spent broth from the erythromycin fermentation contains cell debris and the end-products of microbial metabolism and is either disposed of or concentrated and sold as an animal feed supplement. More recently, enzymatic hydrolysis of the spent broth has been used as a way to further enhance its animal growth-promoting properties (Fidler et al. 2003a, b; Kidd et al. 2003). In this study, we investigated the possibility of recovering a high-value coproduct from the spent broth: soy isoflavones. The project required that coproduct recovery not have a negative impact on the production of the primary product, erythromycin A. By narrowing our search to the spent broth at the end of the fermentation, we ensured that we would not have to alter the manufacturing process for erythromycin in any way. Defatted soybean meal (that which remains of the soybean after the oil is removed and is referred to here simply as soybean meal) supplies protein and carbohydrates for growth of the antibiotic-producing organisms. However, soybean meal also contains isoflavones, and the ultimate fate of the isoflavone component in the soybean meal at the completion of the fermentation was not known prior to our studies. Isoflavones, also referred to as phytoestrogens for their estrogenic activity (Virk-Baker et al. 2010), have commercial value as nutraceuticals and as specialty chemicals used in biomedical research (Dijsselbloem et al. 2004). Since a typical commercial fermentor will consume over 3 metric tons of soybean meal per week and since isoflavones represent about 0.1 to 0.2 % of the soybean meal by weight (Swanson et al. 2004; Murphy and Hendrich 2002), about 3–6 kg of isoflavones is passaged through a single fermentor per week. Multiplying this number by the number of fermentors per plant and the number of soybean-based fermentation manufacturing plants around the world, the total scale of the potential recovery in terms of quantity can be estimated. Additionally, since the value of purified isoflavones can be as much as 10 times that of erythromycin, there is a significant economic incentive to pursue recovery of this coproduct. The main question that we sought to answer in this study was as follows: what happens to the soy isoflavones during the erythromycin fermentation process and can biologically active isoflavones still be recovered at the end of the fermentation?

Many irreversible actinomycete biotransformations of isoflavones have been reported, including hydroxylation, methylation, and chlorination (Chimura et al. 1975; Hosny and Rosazza 1999; Komiyama et al. 1989; Maatooq and Rosazza 2005). It was thought possible that these or other biotransformations might be occurring in the *Saccharopolyspora erythraea* fermentation. Some biotransformations may be desirable in cases where a change in the structure leads to enhanced or altered biological activity (Seo et al. 2011). Our goal,

however, was to harvest a coproduct that fit into an existing commercial market, hence our desire to recover isoflavones—either the glucosides genistin and daidzin or the aglycones genistein and daidzein.

We had shown previously that in soybean-based fermentations, the isoflavone glucosides genistin and daidzin are first hydrolyzed to the aglycones with a β-glucosidase (Hessler et al. 1997; Reeves et al. 2008). We also had preliminary evidence that the aglycones were then further modified, but the nature of the later modification(s) and the enzyme(s) involved were unknown (Reeves et al. 2008).

We speculated that the isoflavone molecules could be irreversibly altered or completely metabolized during the fermentation. Therefore, in order to recover the active isoflavone glucosides or aglycones as coproducts from the erythromycin fermentation spent broth, we initially assumed that identification and inactivation through genetic engineering of genes at one or more steps in the isoflavone biotransformation pathway of *Saccharopolyspora erythraea* (Hessler et al. 1997) would be required. As we discovered from this study, however, these genetic manipulations are unnecessary because the majority of the genistein and daidzein added to the fermentation is not irreversibly modified. Thus, the isoflavone recovery process proposed here is one that would circumvent both the need for genetic engineering of the erythromycin-producing organism and the need for modification of any other aspect of the erythromycin production process.

Materials and methods

Strains and growth media

The erythromycin-producing strain used for this study was the wild-type "white" *Saccharopolyspora erythraea* FL2267. FL2267 is a derivative of ATCC11635 (American Type Culture Collection; Manassas, VA, USA)—the erythromycin-producing strain from which many industrial strains have been developed. FL2267 is a wild-type revertant obtained by eviction of a non-mutagenic integrated plasmid. The plasmid transformation and eviction process was performed to improve the suitability of *Saccharopolyspora erythraea* FL2267 for transformation (Reeves et al., 2007). *Saccharopolyspora erythraea* seed cultures were prepared in CFM1 broth (carbohydrate-based fermentation medium; Reeves et al. 2006), and SCM broth (Reeves et al. 2004) was used for isoflavone feeding experiments where growing cells were used. Both CFM1 and SCM contain Bacto soytone (Difco Laboratories, Sparks, MD, USA) in place of soybean meal, which contains only trace levels of isoflavones. Stirred-jar fermentations were performed in OFM1 broth (oil-based fermentation medium; Reeves et al.

2006). The five additional *Streptomyces* strains used in this study are listed in Table 1.

Chemicals and biochemicals

Genistein, daidzein, genistin, daidzin, and robinin were purchased from Indofine Chemicals (Hillsborough, NJ, USA). Rhamnosylgenistein standards were prepared at Fermalogic, Inc by biotransformation of genistein from Indofine Chemicals. The enzyme α-glucosidase was type I from Baker's yeast (Sigma Chemicals, St. Louis, MO, USA). β-glucosidase (from almonds) was purchased from Sigma. β-glucosidase (from fungi), β-glucanase, and pectinase were industrial grade enzymes from Bio-Cat (Troy, VA, USA). Validase X (hemicellulase and xylanase), Validase® BG (endo β-1,4 glucanase), and Crystalzyme® PML-MX (a mixture of cellulases, hemicellulases, pectinases, and arabinases) were industrial enzyme solutions purchased from Valley Research (South Bend, IN, USA).

Chromatographic analyses

Analytical thin-layer chromatography (TLC) was performed on Silica Gel 60 F_{254} plates (Macherey-Nagel), 0.2 mm thickness. Plates were illuminated at 306 nm using a UVP transilluminator. The primary solvent system used was chloroform/methanol/water (80:20:2). Standards were prepared at 0.3 mg/ml in ethyl acetate (Indofine, Hillsborough, NJ, USA). High-pressure liquid chromatography (HPLC) analysis was performed on Hitachi instrument using Alltech Prevail C18 column 250 mm×4.6 mm. Conditions for the two-component mobile phase system were as follows: mobile phase A: 0.1 % formic acid in H_2O; mobile phase B: 0.1 % formic acid in acetonitrile. The gradient used was 0 min, 75 %A and 25 %B; 10 min, 35 %A and 65 %B; and 20 min, 75 %A and 25 %B. Preparative HPLC purification of compound Gbp1, GC/mass spectral analyses, and NMR analyses were performed under contract by Decode Genetics Analytical Chemistry Services (Lemont, IL, USA).

Washed-cell method for genistein and daidzein feeding experiments

SCM medium (4 ml) was seeded with 5 μl of *Saccharopolyspora erythraea* spores (1×10^8 spores/ml, stored at −80 °C) and incubated at 32 °C for 48 h with shaking at 350 rpm. Of the 4-ml seed culture, 2.5 ml was used to inoculate 25 ml of SCM medium in 250-ml flasks which were incubated at 32 °C for 48 h. The cells were harvested by centrifugation, washed in 25 ml of 50 mM potassium phosphate buffer (pH 7), resuspended in 20 ml of the same buffer, and incubated for 4 h after addition of 20 μg/ml of genistein or daidzein (Indofine Chemical Company). The reaction was stopped by centrifugation of the cells and extraction of the biotransformation products from the supernatant with 20 ml ethyl acetate. The reaction products were concentrated to a final volume 300 μl by evaporation.

Extraction and analysis of unhydrolyzed fermentation broth samples for thin-layer chromatography

A 750-μl broth sample was transferred to a microfuge tube and extracted with 500 μl of ethylacetate/n-butanol (9:1).

Table 1 Summary of genistein and daidzein coproduct potential of industrial antibiotic fermentations shown in Fig. 5

Species	Primary fermentation product	Deglycosylation[a]	Conversion of aglycones[b]	Genistein coproduct potential[c]	Daidzein coproduct potential[c]
S. Reference standards					
C. Uninoculated media		No	No	Yes	Yes
1. *Saccharopolyspora erythraea* ATCC 11635	Erythromycin (antibiotic)	Yes	Yes	Yes	Yes
2. *Streptomyces avermitilis* ATCC 31272	Avermectin (anti-parasitic)	Yes	Yes	Yes	Low
3. *Streptomyces cinnamonensis* ATCC 15413	Monensin (animal growth promotant)	Yes	Yes	Yes	Yes
4. *Streptomyces glaucescens* NRRL B2899	Tetracenomycin (anti-cancer agent)	Yes	Yes	Yes	Very low
5. *Streptomyces hygroscopicus* ATCC 29253	Rapamycin (immunosuppressant)	Yes	Yes	Yes	Yes
6. *Streptomyces rimosus* ATCC 23955	Tetracycline (antibiotic)	Yes	Yes	Yes	Yes

[a] Strain has the ability to deglycosylate isoflavone glucosides (genistin and daidzin) to produce isoflavone aglycones (genistein and daidzein)

[b] Strain has the ability to biotransform the isoflavones genistein and daidzein to other biotransformation products

[c] Potential after acid or enzyme hydrolysis

The solvent extract was evaporated to dryness and resuspended in 25 μl of acetonitrile. A TLC plate was spotted with 5 μl of each concentrated extract.

Acid hydrolysis of fermentation broth samples and analysis by thin-layer chromatography

To 500 μl of fermentation broth in a 15-ml polypropylene test tube with screw cap closure, 250 μl of concentrated (12 N) hydrochloric acid was added. The tube was incubated at 80 °C for 3.5 h and cooled, and 2 ml of water was added to the acidified solution. The aqueous mixture was extracted once with 1.5 ml of ethylacetate/n-butanol (9:1). The solvent layer was evaporated, and the remaining small residue was resuspended in 25 μl of ethyl acetate. Five μl of the resuspended product was spotted on thin-layer silica gel plates which were developed and visualized.

Enzymatic hydrolysis of spent broth for unmasking isoflavone biotransformation products and the generation of genistein and daidzein

The pH of a 3-ml aliquot of thawed culture broth was adjusted to pH 3.0 with sulfuric acid and incubated at 80 °C for 3.5 h. The sample was cooled to 45 °C, and two enzymes were added, Validase® BG (β-glucanase) and Crystalzyme® PML-MX (pectinase/cellulase) (30 μl each of a 1 % stock solution) and incubated for 17 h. The reaction was extracted with 3 ml of ethyl acetate/butanol (9:1), transferred to two microfuge tubes and dried in vacuo. The residue was resuspended in 50 μl of ethyl acetate. Five μl was spotted per lane.

Production and purification of compound Gbp1 (rhamnosylgenistein)

To produce rhamnosylgenistein by biotransformation of pure genistein, 5 μl of a dense spore suspension (1×10^8 spores/ml) of *Saccharopolyspora erythraea* was added to 40 ml of SCM broth in a 250-ml shake flask, and the flask was incubated at 30 °C and 400 rpm (1 in. orbital displacement) for 2 days. The entire 40-ml culture was transferred into a 2-l shake flask containing 100 ml of SCM and 100 mg of genistein (fed directly to the medium from the vial), and the cells were incubated at 30 °C with shaking for 5 to 24 h. The broth was stored at 4 °C until several batches were ready for purification.

To purify rhamnosylgenistein from the fermentation broth, the cells were first removed by centrifugation. The broth was then extracted twice with one-half volume of ethyl acetate, and the solvent layers were combined and concentrated by evaporation under vacuum. The concentrated extracts were then applied to the top of a 12 g silica-gel flash chromatography column and processed on an Isco, Inc. Combi-Flash

Companion Flash chromatography apparatus using a linear gradient elution method programmed to run from a starting condition of 25 % hexane and 75 % ethyl acetate to a finishing condition of 100 % ethyl acetate over a 15-min run. The individual fractions containing pure rhamnosylgenistein were identified by thin-layer chromatography as spot Gbp1. They were combined and dried completely in vacuo. The dried powder was then suspended in 80 % ethanol, heated to 90 °C, and dissolved completely, followed by cooling at 4 °C. Crystals appeared over the next several days and were washed with cold ethanol and dried in vacuo.

Diagnostic enzyme treatment of purified genistein-7-O-α-rhamnoside

Five μl of a 6-mg/ml solution of genistein-7-O-α-rhamnoside in ethanol was added to 100 μl of distilled water. Two μl of a 20-mg/ml stock solution of enzyme was added to the compound, and the mixture was incubated for 1 h at 45 °C. The reaction was dried in vacuo and resuspended in 20 μl of ethyl acetate. Five μl was spotted per lane. The source of the enzymes used is given above.

X-ray crystallography

For X-ray structure analyses, the crystals were mounted onto a 0.3-mm cryo-loop, and data collection was performed at 193 K using graphite monochromated CuK$_\alpha$ radiation ($\lambda = 1.54178$ Å) with a BRUKER-AXS Kappa APEX II diffractometer. Cell refinements and data reductions were carried out with SAINT in APEX2 (Bruker APEX2 Version 2009.5–1 and SAINT version 7.34a Data Collection and Processing Software; Bruker Analytical X-Ray Instruments, Inc.: Madison, WI, USA). Numerical face-indexed absorption corrections were applied using SADABS (Sheldrick 2008a). Structure determinations were carried out by direct methods with XS, and least-squares refinements were carried out with XL of the SHELX (Sheldrick 2008b) package. All diagrams were drawn with 35 % probability thermal ellipsoids. Crystallographic details are given in Table 2. Additional data are in Supplementary Tables S1–S5.

Crystallographic data (excluding structure factors) for the structures of compound Gbp1 reported in this paper have been deposited with the Cambridge Crystallographic Data Center [CCDC 899254]. Copies of the data can be obtained free of charge on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: (international) +44–1223/336–033; e-mail: deposit@ccdc.cam.ac.uk].

Stirred-jar fermentation method

The seed culture was prepared in 25 ml CFM1 broth in shake flasks. Seven shake flasks were prepared per

Table 2 Crystal data and structure refinements for Gbp1 (genistein-7-O-α-rhamnoside)

Temperature	193(2) K	
Wavelength	1.54178 Å	
Formula weight	434.39	
Crystal system	Monoclinic	
Space group	P 2₁	
Unit cell dimensions	a=8.5633(6) Å	α=90°
	b=6.8692(5) Å	β=95.762(5)°
	c=17.1363(12) Å	γ=90°
Volume	1,002.92(12) Å³	
Z	2	
Density (calculated)	1.438 Mg/m³	
Absorption coefficient	0.986 mm⁻¹	
$R(F)^{a}$	0.0348	
$R_w(F^2)^{b}$	0.0829	

[a] $R(F) = \sum \|F_o\| - |F_c\| / \sum |F_o|$ for $F_o^2 > 2\sigma(F_o^2)$

[b] $R_w(F^2) = \left\{ \sum \left[w(F_o^2 - F_c^2)^2 \right] / \sum wF_o^4 \right\}^{1/2}$ for all data. $w^{-1} = \sigma^2 (F_o^2) + (0.048P)^2 + 0.0038P$, where $P = [(F_o^2) = 2(F_o^2)]/3$ for $F_o^2 \geq 0$; $w^{-1} = \sigma^2(F_o^2) \, F_o^2$

fermentor. The seed cultures were inoculated with 15 μl of 2-week-old spores that had been stored in glycerol and frozen at −80 °C. The flasks were incubated at 32 °C while shaking at 280 rpm with a 1-in. circular displacement and grown for 40 h. The fermentation was performed in New Brunswick BioFlo 110 modular benchtop fermentors, using BioCommand Plus software Revision A 2002. The fermentors were filled with 2 l of sterile OFM1 broth and inoculated with 100 ml of seed culture. One hundred ml of a 10 % solution of sterile glucose was also added to the culture medium at this point. Crude soy oil was added to the fermentation starting at 12 h post-inoculation at a rate of 3.3 ml/min. Samples of the fermentation broth were taken at regularly scheduled intervals throughout the course of the 5-day fermentation. The samples were flash frozen in a −80 °C ethanol bath and then stored at −20 °C. Fermentations were performed at 32.5 °C, minimum agitation at 600 rpm, cascaded to maintain dissolved oxygen at above 20 % for approximately 5–6 days. At the completion of the fermentation, the cultures were streaked on E20A agar plates to confirm that the fermentations were not contaminated.

Results

Evidence of isoflavone biotransformation from the erythromycin fermentation

A laboratory-scale fermentation, modeled after the commercial *Saccharopolyspora erythraea* erythromycin fermentation, was performed in 2-l stirred-jar fermentors. Broth samples were taken from the fermentors, extracted with ethylacetate/n-butanol (9:1), concentrated, and analyzed by TLC. The sample time points were (1) 0 h—before the start of the fermentation, (2) 12 h into the fermentation, and (3) 120 h—the spent broth at the completion of the fermentation on day 5 (Fig. 1a).

At 0 h, the two most prominent bands were genistin and daidzin (labeled "g" and "d," respectively). This was not unexpected because these glucosides are known to be the predominant isoflavones in soybean meal (Fig. 1a, 0 h). At 12 h, the isoflavone aglycones, genistein, and daidzein were most prominent ("G" and "D"). This was also not unexpected because genistein and daidzein are created from genistin and daidzin by enzymatic hydrolysis during the early hours of the *Saccharopolyspora erythraea* fermentation (Fig. 1a, 12 h; Hessler et al. 1997; Reeves et al. 2008). However, in the spent broth (120 h), none of the four isoflavone structures from the early fermentation were visible. Instead, a new collection of unknown UV-absorbing compounds appeared (Fig. 1a, 120 h). In order to better determine which of the compounds in the 120-h sample were related to isoflavones, feeding experiments using single purified isoflavones were performed in non-soybean meal containing liquid media.

Evidence of genistein biotransformation from feeding experiments

Feeding experiments were performed to better understand the biotransformation process in more controlled conditions. The results of the genistein and daidzein feeding experiments (Fig. 1b) showed that genistein was rapidly converted into a slower moving product, genistein biotransformation product no. 1 (Gbp1), and similarly, daidzein was converted to Dbp1. Dbp1 moved slightly slower than Gbp1, and therefore, it appears below it on TLC (Fig. 1b), similar to the way daidzein moves slightly slower than genistein, and daidzin moves similarly slower than genistin on TLC (Fig. 1a). Also, Dbp1 matches band bp3 from the spent fermentation broth (120 h, Fig. 1a).

Continuing with the second stage of the feeding experiment, compound Gbp1 was produced and purified in larger amounts, and then fed to *Saccharopolyspora erythraea* cells. A new slower moving spot, Gbp2, was identified. In this experiment, Gbp1 was completely biotransformed into Gbp2 after 24 h (Fig. 1c). In reference to the previous experiment, compound Gbp2 matched up in mobility on TLC (Fig. 1c, 24 h) with band bp1 from the unhydrolyzed spent fermentation broth (Fig. 1a, 120 h). Both bands Gbp2 and bp1 traveled half-way between the robinin (R) and daidzin (d) reference standards (Fig. 1a, 120 h).
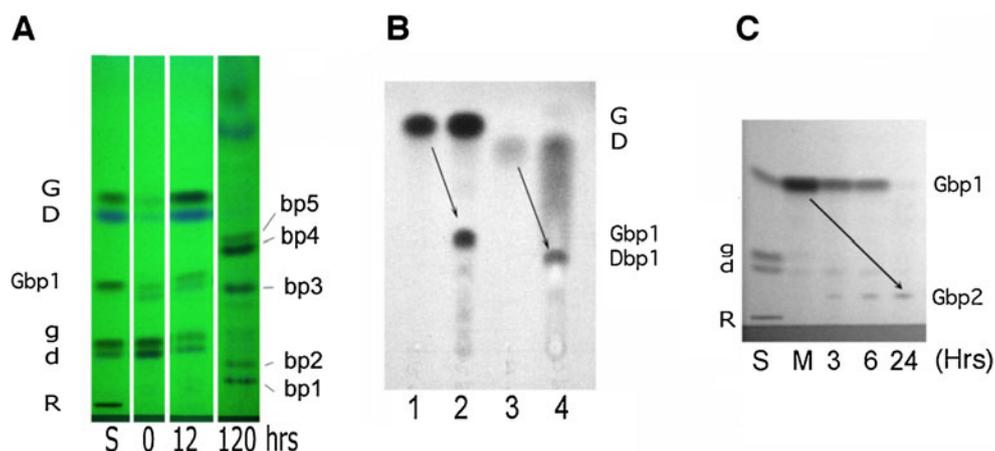
Fig. 1 The isoflavone biotransformation process analyzed by thin-layer chromatography. **a** Extracts from three different time points (0, 12, and 120 h) during the fermentation. **b** Feeding experiment where genistein and daidzein were fed to washed cells of *Saccharopolyspora erythraea*. *Lane 1*, genistein reference standard; *lane 2*, extract from the genistein reaction after 4-h incubation at 32 °C; *lane 3*, daidzein reference standard; *lane 4*, extract from the daidzein reaction after 4-h incubation at 32 °C. Solvent system, hexane/ethylacetate/methanol (20:20:8). *Arrows* indicate the direction of biotransformation from the isoflavone aglycones to their first biotransformation products. **c** Biotransformation of genistein biotransformation product 1 (Gbp1). Gbp1 was reacted with *Saccharopolyspora erythraea* growing in SCM broth

for the indicated period of time. *Lane S*, reference standards; *lane M*, medium sample prior to the start of incubation. *G* genistein, *D* daidzein, *Gbp1* genistein biotransformation product 1, *Gbp2* genistein biotransformation product 2, *Dbp1* daidzein biotransformation product 1, *g* genistin, *d* daidzin, *R* robinin (an unrelated isoflavone used as a standard for its three sugar groups). The plates, containing a fluorescence indicator, were developed in chloroform/methanol/water (80:20:2) and photographed under ultraviolet light illumination. A Dbp1 reference standard is not included in *lane S*; however, Dbp1 can be seen in *lanes 0 h* and *12 h*, paired up with Gbp1 which migrates just above it

Analysis of fermentation broth and isoflavone biotransformation products by acid and enzyme hydrolysis

In order to help determine whether bp1 and bp2 from the 120-h sample were related to the isoflavones, they were copurified by flash chromatography and subjected to acid hydrolysis (Fig. 2b). At the high acid concentrations (1-, 2-, and 4 N), the only visible products of the reaction were genistein and daidzein, which confirmed the connection of these biotransformation products to the isoflavone parent compounds. Lower acid concentrations (0.05- and 0.1 N) revealed the first-stage conversion products, Gbp1 and Dbp1, which also confirmed a connection with the first-stage biotransformation products.

In a related experiment when fermentation samples were analyzed, all three time points gave similar looking hydrolytic profiles, that is, genistein and daidzein were the main products regardless of what time the samples were taken (Fig. 2a). The identity and concentrations of genistein and daidzein in these hydrolyzed samples were confirmed by HPLC. The levels of genistein and daidzein at the end of the fermentation were found to be 100 % (±10 %) and 67 % (±3 %) of their starting levels. The structures of the glucosylated isoflavones genistin and daidzin and the structures of their hydrolytic products genistein and daidzein are shown (Fig. 2d).

In a related work, purified compound Gbp1 was subjected to hydrolysis by commercial glycosidic enzyme preparations, revealing its susceptibility to these enzymes

(Fig. 2c). Three enzyme preparations showed reactivity with Gbp1, and each of the active enzyme mixtures converted Gbp1 back to genistein (G). These reactions were not run to completion, and therefore, they show both compounds. The three active enzyme preparations were a mixture of fungal α- + β-glucosidase from Bio-Cat (lane 2), a pectinase from Bio-Cat (lane 3), and crystalzyme from Valley Research (lane 4). These industrial preparations contained crude mixtures of enzymes that could hydrolyze a wide variety of glycosidic bonds.

Compound Gbp1 was determined to be genistein-7-O-α-rhamnoside (rhamnosylgenistein)

In order to determine the structure of Gbp1, larger amounts of the compound were produced, purified by flash chromatography, and analyzed by HPLC, high-resolution mass spectrometry, COSY NMR, and X-ray crystallography. Compound Gbp1 appeared as a single peak and eluted just prior to daidzein on HPLC (Fig. 3a). Using mass spectrometry, the molecular ion of compound Gbp1 in Electrospray ionization (ESI)-positive mode was found to be 417. Coupling with the ESI-negative results of molecular ion at 415, the molecular weight of compound Gbp1 was determined to be 416 (Fig. 3b). Tandem mass spectrometry fragmentation analysis was carried out to compare genistein (m/z 271), genistin (m/z 433), and compound Gbp1. The fragmentation pattern of m/z 271 from compound Gbp1 matched the pattern of m/z 271 from
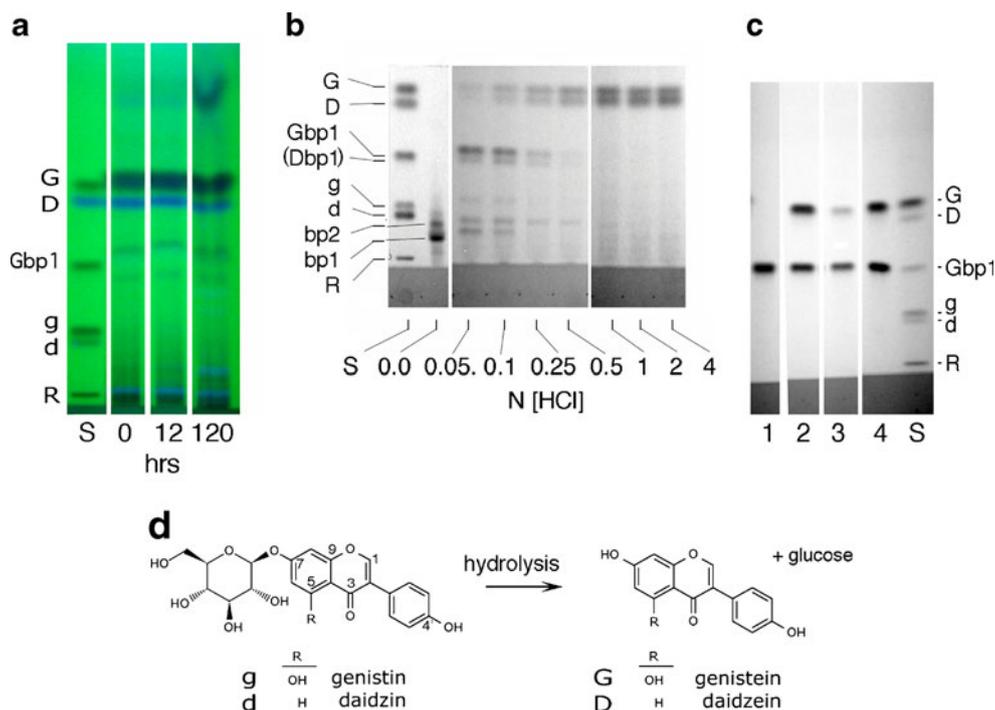
Fig. 2 Acid and enzyme hydrolysis of fermentation samples and purified compounds from the fermentation. **a** Extracts from the three time points after strong acid hydrolysis. **b** Hydrolysis of isoflavone biotransformation products bp1 and bp2 that were purified from the spent broth of an OFM1 stirred-jar fermentation. Acid concentrations ranged from 0 N to 4 N HCl. Acid treatment was at 80 °C for 0.5 h. *S* reference standards, *G* genistein, *D* daidzein, *Gbp1* genistein biotransformation product 1, *Dbp1* daidzein biotransformation product 1, *g* genistin, *d* daidzin, *R* robinin. A reference standard for Dbp1 was not available, but its migration position can be best observed in *lane 0.1 N* genistin (Fig. 3b, inset). Based on the above results, the full structure of genistein must be present within compound Gbp1 as it is within genistin.

just beneath Gbp1. The plates were developed in chloroform/methanol/water (80:20:2). **c** Extracts from reactions of industrial enzymes with purified compound Gbp1. *Lane 1*, untreated Gbp1; *lane 2*, a mixture of fungal α-glucosidases and β-glucosidases; *lane 3*, Bio-Cat pectinase; *lane 4*, Crystalzyme® PML-MX (a mixture of cellulases, hemicellulases, pectinases, and arabinases). **d** Isoflavone acid or enzyme hydrolysis showing chemical structures. *S* reference standards, *G* genistein, *D* daidzein, *Gbp1* genistein biotransformation product 1, *g* genistin, *d* daidzin, *R* robinin. The plate was developed in chloroform/methanol/water (80:20:2)

Finally, compound Gbp1 was crystalized from 80 % ethanol and subjected to X-ray crystallographic analysis. Its structure was determined to be genistein-7-O-α-rhamnoside (IUPAC name: 5-hydroxy-3-(4-hydroxyphenyl)-7-(((2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)-4H-chromen-4-one) (Fig. 4a; Table 2; Supplemental Information Tables S1–S5). The rhamnosyl group is shown as the natural L isomer (an assumption at this point) and is attached by an α-linkage to the core isoflavone structure at C-7, whereas in genistin, a glucosyl group is linked at the same C-7 location by a β-linkage. Structures of the known intermediates in the genistin biotransformation pathway are shown (Fig. 4b).

Acid hydrolysis of five additional industrial actinomycete fermentations reveals hidden isoflavone content

Five additional actinomycete species of commercial importance were compared to *Saccharopolyspora erythraea* for their isoflavone coproduct potential. The results (Fig. 5; Table 1) showed all five fermentations to have at least some isoflavone coproduct potential, particularly for genistein recovery. Daidzein was absent in the case of *Streptomyces glaucescens* (Fig. 5b, lane 4) and weak for *Streptomyces avermitilis* (Fig. 5b, lane 2). The results also show that it is impossible to predict from the analysis of the unhydrolyzed spent broth whether a strain will have good coproduct potential or not. Comparing the *Streptomyces glaucescens* and *Streptomyces rimosus* profiles before hydrolysis, both have nearly blank profiles with no bands (Fig. 5a, lanes 4 and 6), indicating complete biotransformation or destruction of the isoflavones. After hydrolysis, *Streptomyces rimosus* had strong bands for genistein and daidzein (Fig. 5b, lane 6), whereas *Streptomyces glaucescens* had a weak band for genistein and a very weak band for daidzein (Fig. 5b, lane 4). The results suggest that *Streptomyces glaucescens* has a biotransformation process that involves irreversible biotransformation or destruction of the isoflavones, whereas *Streptomyces rimosus*, like *Saccharopolyspora erythraea*, has a reversible masking pathway for the majority of the isoflavone material in the fermentation.

Fig. 3 **a** HPLC analysis of compound Gbp1 with three isoflavone reference standards, genistin, daidzein, and genistein. **b** Mass spectral analysis of compound Gbp1 (*large chart*). Comparative mass spectral analysis of genistin (*top*) with compound Gbp1 (*bottom*) in the fragment m/z 271 (*inset charts*)
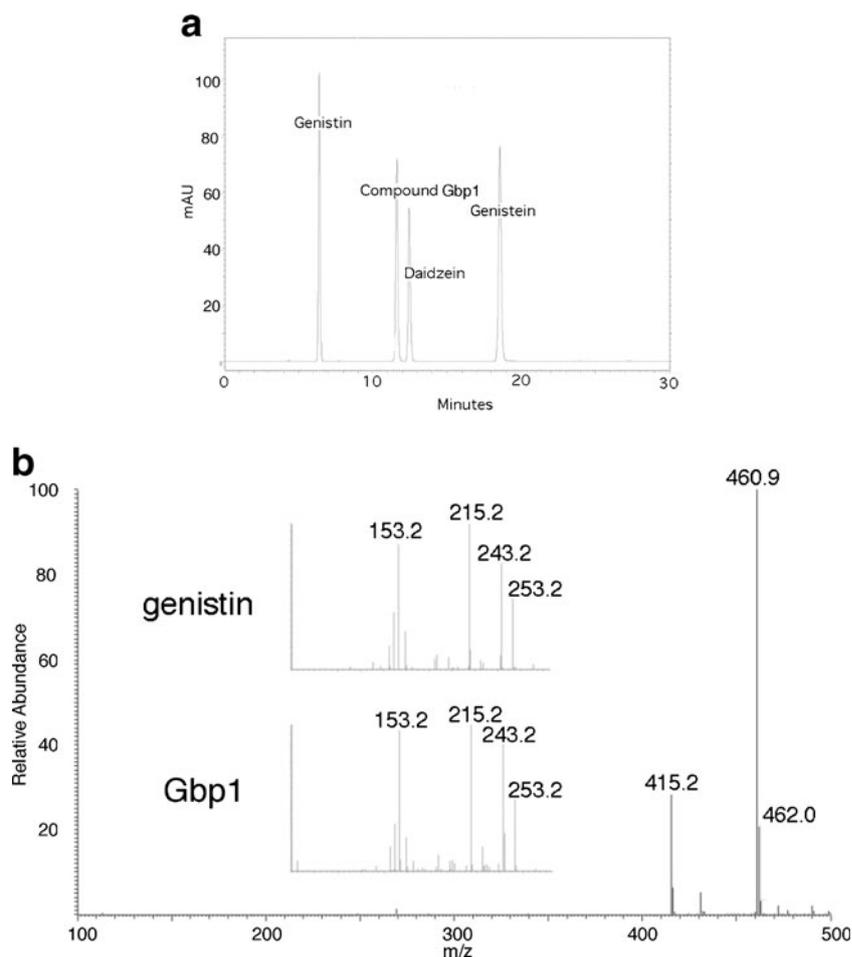
Other ways where industrial actinomycetes can vary are in the type of biotransformation products they produce. *Streptomyces cinnamonensis* (Fig. 5a, lane 3), for example, showed four new UV absorbing bands, different from those seen previously with *Saccharopolyspora erythraea*. All of these presumed biotransformation products, however, could be converted back to genistein and daidzein by acid hydrolysis (Fig. 5b, lane 3).

These results show that *Saccharopolyspora erythraea* is not unique in its ability to biotransform isoflavones and that commercially useful isoflavones should also be recoverable from other industrial fermentations following acid or enzyme hydrolysis of their spent broth.

Discussion

As a result of this study, we showed that it is possible to efficiently recover isoflavone coproducts from laboratory-scale industrial antibiotic fermentations. We initially assumed that this would require genetic engineering to block the isoflavone biotransformation process, but this was discovered not to be necessary. Although it is true that isoflavones are

highly altered by *Saccharopolyspora erythraea* in a multistep biotransformation process, the modifications can be allowed to proceed because they can be reversed at the end of the fermentation. The spent broth was chosen as the source of coproducts since harvesting at the end of the fermentation, rather than at an earlier time point, ensures that the coproduct recovery process will not possibly interfere with the production of the primary product, erythromycin A. When dealing with an industrial process of this magnitude, it is highly desirable that interference with the existing process be held to a minimum, and two advantages of this system are that neither the existing erythromycin manufacturing process nor the producing organism need to be altered.

Our results showed that the first stage of genistein biotransformation is characterized by the rhamnosylation of the C-7 hydroxyl group. Evidence for a second, and possibly final, stage of genistein's biotransformation process was also obtained, and the chemical and biological characterization of later biotransformation product(s) is currently under investigation, and it will be reported elsewhere.

That a rhamnosylase was involved in the biotransformation pathway led to attempts to genetically block this step; however, this approach was abandoned, after extensive

Fig. 4 a Compound Gbp1, genistein-7-O-α-rhamnoside, determined from X-ray crystallographic analysis (see supplemental information). **b** *Saccharopolyspora erythraea* biotransformation pathway for genistin. The C-5 hydroxyl (*gray shading*) is replaced with hydrogen in the daidzein molecule. **c** *Saccharopolyspora erythraea* biotransformation of flaviolin (23)
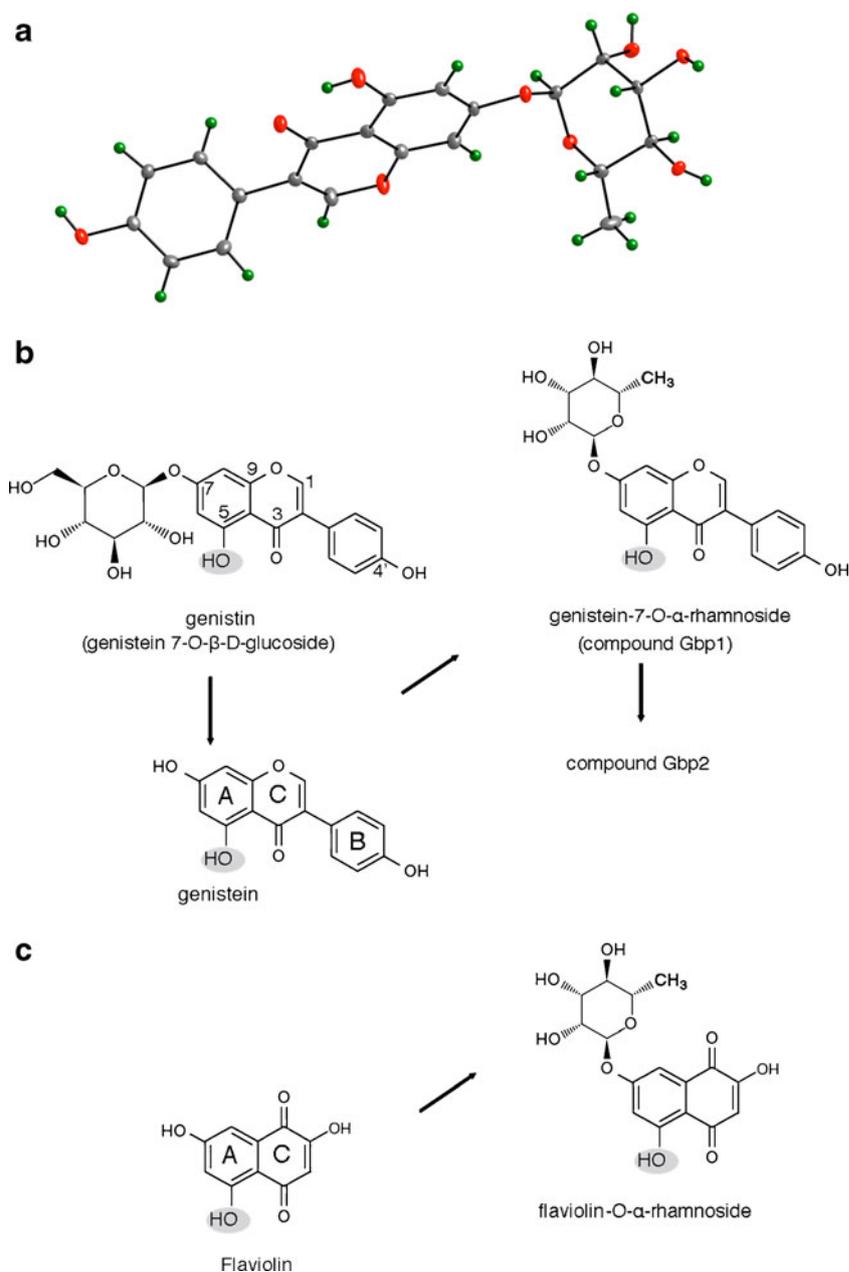
efforts proved unsuccessful (I. A. Brikun, A. R. Reeves, and J. M. Weber, unpublished data). This approach, had it been technically possible, would have required that a new genetically engineered modification be put into each industrial production strain, and this we predict would be less appealing to industry than the approach we propose here.

Glycosylation is a mechanism used to confer resistance to antibiotics used by some actinomycetes (Quirós et al. 1998), and glycosylation is not an uncommon microbial biotransformation reaction. However, some insight into why this biotransformation occurs to isoflavones can be found from a previous report which describes the rhamnosylation of another

phenolic compound, flaviolin, by *Saccharopolyspora erythraea* (Cortes et al. 2002). Flaviolin is a natural product involved in the formation of the red pigment that is biosynthesized by *Saccharopolyspora erythraea* and is nearly identical to the A and C rings of the isoflavone core structure (Fig. 4c). Furthermore, the rhamnosyl group is transferred to the equivalent oxygen atom on each structure. Therefore, genistein is highly likely to be acted upon by the same rhamnosylase that biotransforms flaviolin. The production of flaviolin and rhamnosylflaviolin by *Saccharopolyspora erythraea* could explain the appearance of the additional spots seen in the spent broth sample extract, particularly bp4 and bp5 (120 h, Fig. 1a).
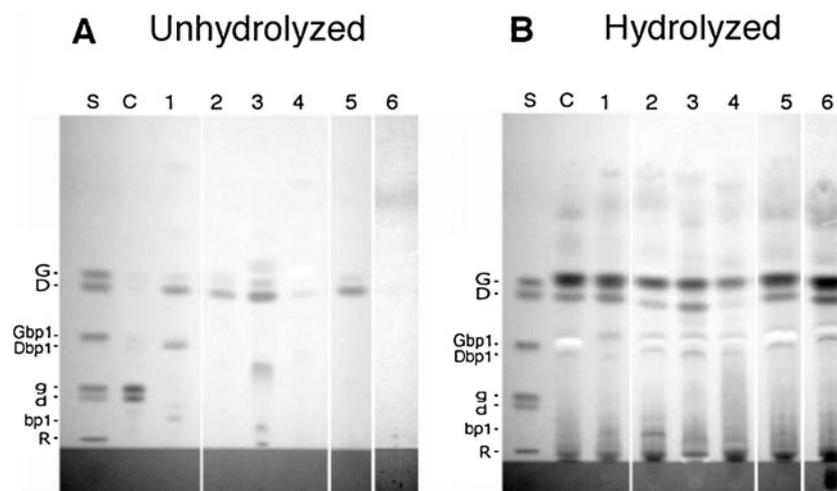
Fig. 5 Analysis of extracts from a collection of antibiotic fermentations before (*left panel*) and after (*right panel*) acid hydrolysis of the spent broth. *S* isoflavone reference standards, *C* uninoculated OFM1 media containing soy flour, *1 Saccharopolyspora erythraea* ATCC 11635 (erythromycin), *2 Streptomyces avermitilis* ATCC 31272 (avermectin), *3 Streptomyces cinnamonensis* ATCC 15413 (monensin), *4 Streptomyces glaucescens* NRRL B2899 (tetracenomycin), *5*

Streptomyces hygroscopicus ATCC 29253 (rapamycin), *6 Streptomyces rimosus* ATCC 23955 (tetracycline). *S* reference standards, *C* OFM1 medium control, *G* genistein, *D* daidzein, *Gbp1* genistein biotransformation product 1, *Dbp1* daidzein biotransformation product 1, *bp1* biotransformation product 1, *g* genistin, *d* daidzin, *R* robinin. The plates were developed in chloroform/methanol/water (80:20:2)

In reference to the commercial applications of this study, it is theoretically likely that our results will extrapolate to large-scale commercial fermentors using high producing strains. This is because, although commercial strains will contain hundreds of different mutations, it is likely that the vast majority of them would confer a loss of biochemical or genetic function rather than a gain of function. Therefore, the commercial strains should show equal or less

biotransforming ability than the wild-type strain, rather than more. That said, each different manufacturing process could have a variable influence on the biotransformation pathway, and therefore, each strain and process will have to be tested separately to answer this question. A basic flow diagram for an idealized erythromycin fermentation process including isoflavone biotransformation and coproduct recovery is shown (Fig. 6).
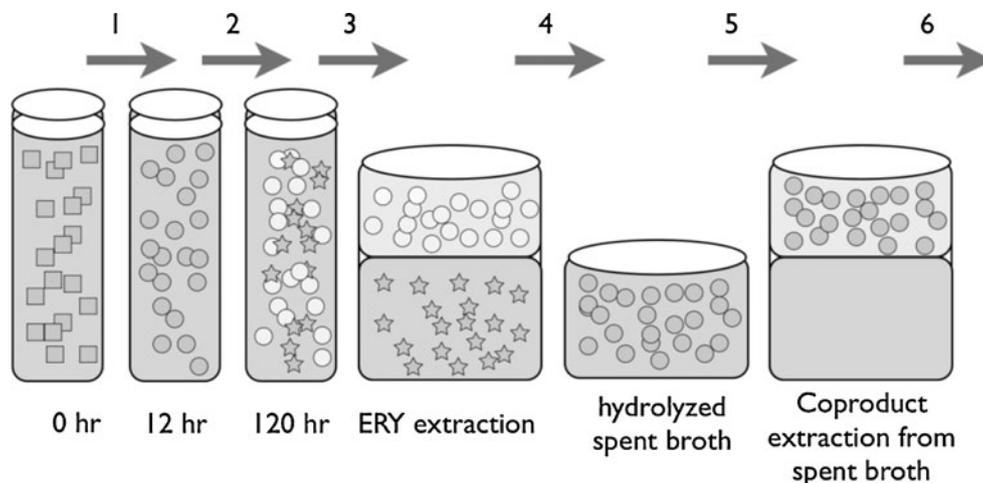
Fig. 6 Basic outline of a proposed commercial process to produce isoflavone coproducts from a soybean-based antibiotic fermentation. Fermentors are shown at the three time points from which samples were taken for analysis (Fig. 1). At 0 hr the native isoflavone glucosides in soybean meal are shown (squares). By 12 hr the isoflavone aglycones predominate (dark circles). The spent broth, 120 hrs, shows the isoflavone biotransformation products (dark stars) coexisting with erythromycin A (light circles). Following the completion of the

fermentation, the spent broth is shown being transferred to an extraction chamber (*step 3*) at which point the erythromycin (*light gray circles*) is removed. The spent broth is then hydrolyzed (*step 4*) which converts the isoflavone biotransformation products (*stars*) into genistein and daidzein (*dark gray circles*) which are then extracted (*step 5*). The extracted isoflavones can then be further purified, and the remaining spent broth can then be handled as it was before the isoflavones were removed

In conclusion, chemically altered or "masked" forms of genistein and daidzein were found in the spent broth of the erythromycin fermentation (120 h, stars, Fig. 6). Once erythromycin is removed (light gray circles, step 3, Fig. 6), the spent broth can then be hydrolyzed with acid or enzyme treatments to convert isoflavones (stars) to their parental forms, genistein and daidzein (step 4, dark gray circles, Fig. 6), which are then recovered from the treated broth by solvent extraction (step 5, Fig. 6). Hydrolysis releases or "unmasks" isoflavone biotransformation products into their commercially useful parental form. In addition, a preliminary survey revealed that other industrial antibiotic fermentations also have this potential to serve as sources of isoflavone coproducts.

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