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Isolation of isoflavones from soy-based fermentations of the erythromycin-producing bacterium *Saccharopolyspora erythraea*

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Abstract A search for an abundant and economical source of isoflavones, particularly genistein, led to the discovery that the erythromycin-producing organism *Saccharopolyspora erythraea* also produces this promising new cancer-prevention agent. Erythromycin fermentation is a large-scale, soybean-based process used world-wide for the commercial production of this medically important antibiotic. Results from this study indicate that genistin (the glucoside form of genistein), which is added to the fermentation in the soybean media, was converted to genistein through the action of a β -glucosidase produced by the organism. Genistein was co-extracted with erythromycin from the fermentation broth, then separated from erythromycin during the second step of the purification process for the production of erythromycin.

Introduction

Isoflavonoids are natural products found in soybeans and leguminous plants and are currently under intensive study regarding their application to cancer prevention (Barnes et al. 1995) and other potential health benefits (JJ Anderson et al. 1995; JW Anderson et al. 1995). Epidemiological evidence suggests that dietary intake of isoflavonoids may be responsible for the low incidence of breast and colon cancer seen in Asians (Messina and

Barnes 1991). The biochemical mechanisms underlying the positive health effects of isoflavones, particularly genistein, are still being sought. Genistein can be both weakly estrogenic (Levy et al. 1995) and antiestrogenic (Henderson et al. 1982; Verdeal et al. 1980). Other recent studies show that genistein is active against key enzymes involved in carcinogenesis pathways and can protect against chemically induced cancers in vivo (Lamartiniere et al. 1995a,b). Current market demand for genistein comes from its use in biological research as an enzyme inhibitor (Akiyama et al. 1987; Constantinou et al. 1995) and as a chemical probe to explore cellular signal transduction pathways (Messina et al. 1994). Isoflavone extracts from processed soybeans which contain high concentrations of genistein are also used as nutritional supplements by the food industry. Future markets may emerge for its use as a chemical intermediate in the formulation of novel anticancer therapeutics (Uckun et al. 1995).

Our interest in isoflavones stems from the need for a more economical source and an adequate supply of isoflavones, in particular genistein, to be used in biomedical research and in nutritional products. Several other new processes for isoflavone production have appeared recently (Fleury and Magnolato 1992; Ogawara 1986; Shen 1994). Isoflavones are found in nature in the glycosylated form as a minor constituent of soybeans and other leguminous plants. The bioactive aglycone derivative can be formed through enzyme (β -glucosidase) treatment or acid treatment of soybeans, followed by solvent extraction; or the compound can be chemically synthesized (Baker and Robinson 1928; Prakash and Tanwar 1995). Both of these current production methods are costly. We sought a more economical process through the development of a dedicated de novo fermentation process using strains from the American Type Culture Collection (ATCC) (Chimura et al. 1975; Ganguly and Sarre 1970). This approach, though successful at demonstrating isoflavone production, led us to a more significant finding, i.e., that a commercial large-scale fermentation that already exists could provide an

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abundant and economical source of isoflavones as an added-value co-product.

Here we report the isolation of isoflavones, in particular genistein, from the fermentation broth of the erythromycin-producing organism *Saccharopolyspora erythraea* ATCC 11635 (Weber et al. 1996). Results are presented which show that genistein and erythromycin are co-extracted by an organic solvent and are later separated using the procedure described for the industrial production of erythromycin (Bunch and McGuire 1953).

Materials and methods

Strains

Sac. erythraea was purchased from the ATCC (ATCC 11635); this strain is also available from the National Center for Agriculture Utilization Research, Peoria Ill. (formerly the Northern Regional Research Laboratory) as strain number NRRL 2338. Experiments in this study were performed with "white" or original 11635 strain as opposed to the "red" variants of 11635 that form spontaneously from the parent strain at high frequency. The three reported isoflavone-producing Actinomycete species used in this study were: *Streptomyces roseolus* ATCC 31047, *Micromonospora halophytica* subsp. *halophytica* ATCC 27596, and *Micromonospora halophytica* subsp. *nigra* ATCC 33088. All three strains were obtained directly from ATCC.

Media

E20A agar medium (Weber and McAlpine 1991) contained (per liter aqueous solution): 5 g bacto-soytone, 5 g soluble starch, 3 g CaCO_3 , 2.1 g 3-(*N*-morpholino)propanesulphonic acid (MOPS) buffer, and 20 g bacto-agar. (The pH of E20A and the following media was not adjusted prior to inoculation.) Germination broth (Weinstein et al. 1967): beef extract, 0.3%; tryptone, 0.5%; dextrose, 0.1%; soluble starch, 2.4%; yeast extract, 0.5%. G1 broth: the following were added to 980 ml water – NZ amine, 20g; yeast extract, 10 g; soluble starch, 20g; NaCl, 2.5g; CaCO_3 , 3.5g; trace elements, 1 ml; the solution was autoclaved and then 20 ml of 50% glucose was added. Soytone broth: Soytone (Difco), 20 g; yeast extract, 5 g; NaCl, 2.5 g; CaCO_3 , 3.5 g; soluble starch, 20 g; trace elements solution (Hopwood et al. 1985), 0.25 ml. [Note: in some cases different soybean substrates including SoyaFluff (Central Soya), or Nutrisoy Grits (Cargill) were substituted for the soytone as described in the text.] AVMM minimal medium (Weber and McAlpine 1991): asparagine, 5.0 g; K_2HPO_4 , 1 g; KH_2PO_4 , 1 g; MgSO_4 , 0.1 g; FeSO_4 , 0.01 g; agar, 15 g; distilled water, 1 l. Following autoclave sterilization 20 ml of 50% glucose, was added along with 1 ml of trace elements solution (Hopwood et al. 1985).

Shake flask fermentations

Spores were transferred by sterile loop from E20A-agar slants to 3 ml of Tryptic Soy Broth (Difco) in 16 × 125 mm test tubes with fitted plastic caps. Test tube cultures were incubated at 32 °C for 2 days while shaking at 300 rpm at an angle in a cabinet shaker with a 2.54-cm rotary displacement. The test tube culture was transferred to 50 ml of the appropriate medium in a 500-ml Erlenmeyer flask and the flask was incubated for 4–6 days at 32 °C while shaking.

Chemicals and enzymes

Genistein and daidzein were purchased from Indofine, Belle Mead, N.J., USA. The following were purchased from Sigma: erythromycin-A (approx. 98%) (E-6376); β -glucosidase (G-0395)

EC 3.2.1.21 from almonds; and *p*-anisaldehyde (A0519). Solvents were high-pressure liquid chromatography (HPLC) grade from EM Science, Gibbstown, N.J., USA.

High pressure liquid chromatography (HPLC)

Instrument components were purchased from Hitachi. Column: Synchropak RPP-100-25 (Synchrom) reverse phase C18, 100 Å (10 nm), 250 × 4.6 mm. The UV detector was set at 260 nm. Initial conditions were: 50% methanol, 50% 0.1 M ammonium acetate, pH 4.6, followed by a 20-min gradient to 100% methanol, followed by a 15-min 100% methanol wash, then a return to equilibration for 5 min in 50% methanol.

Thin-layer chromatography (TLC)

TLC plates were from Whatman (Clifton, N.J., USA), LHPKF silica gel, 6 nm; layer thickness 200 nm, 10 × 10 cm. Solvent system – chloroform: methanol (10:1); detection system: for genistein, UV 306 nm (UVP transilluminator) and, for erythromycin, anisaldehyde spray followed by heating. Anisaldehyde spray reagent was prepared in a beaker chilled in ice containing 180 ml of 95% ethanol, 10 ml conc. sulfuric acid, 10 ml *p*-anisaldehyde, and 2 ml conc. acetic acid. Plates were sprayed to saturation and then heated from the glass side with a heated air gun. Genistein appears as a dark spot under UV illumination and has a high R_f value in the solvent system described above. Erythromycin-A is not significantly detectable by UV illumination but appears as a blue-gray spot after anisaldehyde spray and has a low R_f value in the solvent system described above.

GC/mass spectral analysis

Mass spectra were obtained using a Finnigan MAT 90 mass spectrometer under the following conditions: injection port temperature, 220 °C; ion source temperature, 250 °C; gas chromatography column, DB5, 30 meter, fused silica capillary programmed from 120 to 230 °C at 5 °C/min; ionizing voltage, 70 eV; resolution, 1000 (10% valley definition). The mass range from m/z 70 to m/z 1000 was scanned at a rate of 1.6 s/decade under the control of a Micro VIP 11/73 computer (US Design, Palo Alto, Calif., USA).

The trimethylsilyl derivatives were prepared using standard conditions (Schram 1991); briefly, approximately 50 μg of sample was dissolved in 40 μl of BSTFA + 1% TMCS (Pierce, Rockford, Ill., USA) and 10 μl of pyridine and heated at 100 °C for 1 h. Then, 1 μl of the reaction mixture was injected onto the GC column.

Topoisomerase assay

The plasmid-linearization assay was performed according to the instructions provided in the manual for Topoisomerase II Drug Screening Kit (TopoGen, Columbus, Ohio, USA). The kit is designed specifically for the identification of Topo II "poisons" such as genistein. This reaction is also described in detail by Constantinou et al. (1995). Each reaction contained the following components from the kit: 2 μl of 10 × buffer, 1 μl of supercoiled pYRG DNA, 3 μl of topoisomerase II. The poisoned reactions contained either 1 μl of authentic genistein (10 mg/ml) (lane 2) or 2 μl of HPLC-purified material (1 mg/ml) (lane 3). A variable amount of water was added to bring each reaction volume to 20 μl . Reactions were incubated for 30 min at 37 °C, then 2 μl of 10% sodium dodecyl sulfate (SDS) was added, followed by proteinase K to a final concentration of 50 $\mu\text{g}/\text{ml}$. The reactions were incubated for another 15 min at 37 °C. Loading buffer (0.1 volume) was added, reactions were then extracted with a chloroform:isoamyl alcohol mixture (24:1) and gently mixed, then microcentrifuged. A 15- μl portion of the blue upper aqueous layer was loaded onto a 1% 1 × TAE agarose gel. The gel was stained with ethidium bromide following electrophoresis.

Results

Genistein production by reported isoflavonoid producers

Three reported isoflavone-producing Actinomycete strains were obtained from the ATCC and analyzed in shake-flask fermentations for isoflavone production. Each strain was grown in seven different media (Materials and methods), including a defined minimal medium (AVMM), two rich media not containing soybeans (Germination Broth, and G1 broth), and three rich media containing soybeans. Results are shown for *S. roseolus* (Fig. 1). For each fermentation the culture broth was harvested at day 4, extracted with ethyl acetate, concentrated by evaporation, and analyzed by HPLC. HPLC analyses (Fig. 1A–D) shows two late-eluting peaks – peak 1, at approximately 12 min, and peak 2, eluting at approximately 15 min – that were present only in the soybean-based media (Fig. 1D). The results shown in Fig. 1D are for a fermentation using Bacto Soytone (Difco); however, media containing SoyaFluff (Central Soya), and Nutrisoy Grits (Cargill) gave similar results. The HPLC effluent responsible for

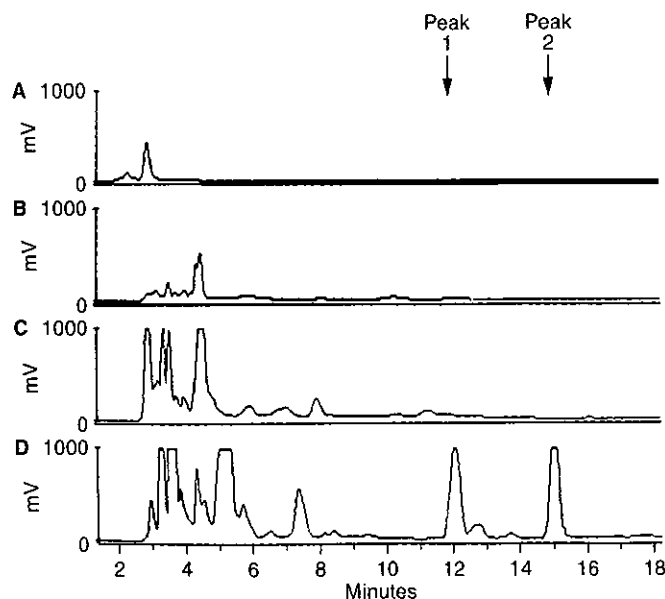


Fig. 1A–D Media-dependent production of genistein by *Streptomyces roseolus*. Fermentations were performed using 500-ml shake flasks containing 50 ml of growth medium, incubated at 32 °C, rotating at 300 rpm, for 4 days. Culture broth was extracted once with an equal volume of ethyl acetate. The solvent extract was concentrated to 100 μ l and 10 μ l was injected into an Hitachi HPLC system equipped with a C-18 reverse phase column. UV absorbance was measured at 260 nm. HPLC elution profiles were obtained for the following growth conditions (see materials and methods for media recipes): **A** minimal medium (AVMM); **B** Non-soy complex medium (Germination broth); **C** non-soy complex medium (G1 broth); and **D** Soytone broth. *Peak 1* and *Peak 2* were later determined to be daidzein and genistein, respectively, by comparison to authentic standards and by GC/Mass spectral analyses (Fig. 2)

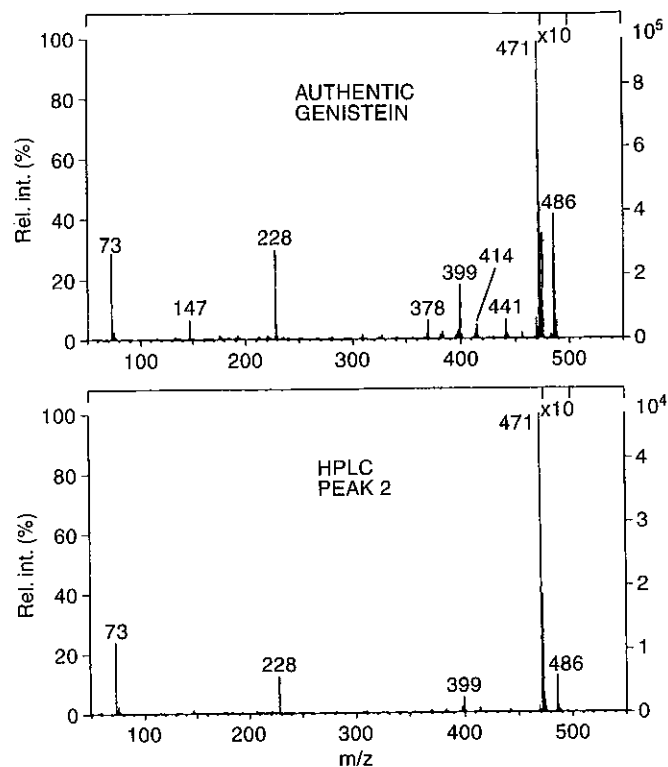


Fig. 2 GC/MS analysis of HPLC-purified fermentation extracts. The mass spectra of the trimethylsilyl derivatives of a reference sample of genistein (*top*) and the HPLC-purified material from peak 2, Fig. 1 (*bottom*). The mass spectra and GC retention times (20:53 min) of both samples were identical

peak 2 was collected, extracted, concentrated, and compared to authentic standards by mass spectral analyses (Fig. 2); the compound eluting at peak 2 was indistinguishable from the isoflavone genistein. Peak 1 was determined to be daidzein by comparison with authentic standards in both HPLC and TLC analyses. Isoflavone production was therefore confirmed this way for all three strains, but only when the reported producers were grown in soybean-based growth media.

An experiment was performed to demonstrate that the genistein found in the extractions described above was not simply extracted from the media. Inoculated and uninoculated soybean-based broths were extracted following 6 days of shaking incubation at 32 °C. Results from HPLC analyses indicate that peaks for genistein were not present in extracts from the uninoculated broth (Fig. 3D); however, genistein was present in the fermentation broth extracts from *S. roseolus* (Fig. 3A), as shown previously. The fermentation was therefore determined to be an essential part of the process for genistein production from soybeans.

Further experiments were performed to determine how the genistein might be formed. Uninoculated broth was treated with purified β -glucosidase (Sigma), extracted with ethyl acetate, and the results compared with those of extracts from actual fermentation broth. Very

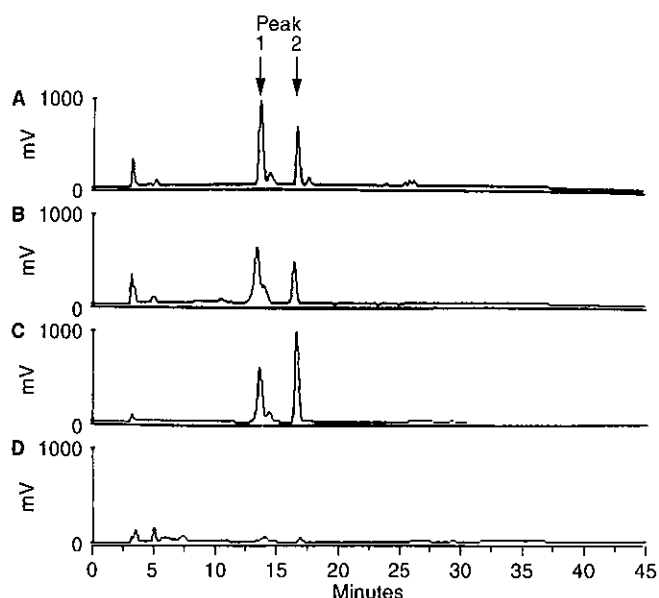


Fig. 3 HPLC analysis of solvent extracts from the fermentation broth of **A** *S. roseolus* and **B** *Sac. erythraea*. Shake flask cultures containing 50 ml of SoyGrits medium in a 500-ml Erlenmeyer flask were inoculated with a loopful of spores from each strain and incubated for 6 days at 32 °C while shaking at 300 rpm. Cells and media components were removed and the broth was extracted once with an equal volume of ethyl acetate. The extract was then evaporated to dryness, resuspended in 100 μ l of ethyl acetate and 1 μ l of this was injected into the HPLC with 9 ml of ethyl acetate carrier. **C** Enzyme (β -glucosidase) treatment: 100 μ l of a 1 mg/ml solution of Sigma β -glucosidase was added to the flask prior to the 6-day incubation. **D** Uninoculated broth. The untreated broth control was subjected to the same 6-day incubation period before extraction, as the other cultures

similar isoflavone production profiles were obtained with both treatments (Fig. 3A, C). This showed that production of genistein during the fermentation could be explained by the simple enzymatic hydrolysis of genistein glucosides in the media (Fig. 4). This result, however, does not rule out the possibility that de novo biosynthesis also contributes to isoflavonoid production under these conditions, but simple bioconversion is sufficient to explain the amount of genistein found in our experiments.

The amount of genistein produced by these fermentations, approximately 2.5–5.0 μ g/ml, was never greater

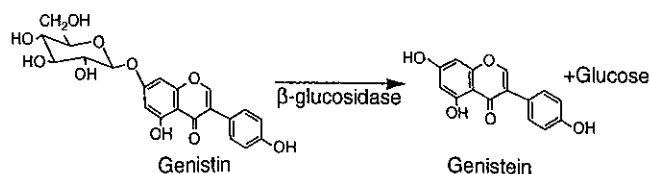


Fig. 4 The hydrolysis of the isoflavonoid glucoside, genistin, to create genistein. Soybeans contain significant quantities of genistein glucosides; including the simple glucoside (*genistin*), the acetylglucoside or the malonylglucoside (Wang and Murphy 1994a). The glucose moiety (or modified glucose moiety) can be removed by acid treatment, or by enzyme treatment with β -glucosidase(s) to create the isoflavone *genistein*

in molar quantities than the amount of genistein glucosides (*genistin*) that was calculated to be present in the soybean media (Wang and Murphy 1994a, b). Instead, it was observed that 5–10 times less genistein was produced from the fermentation at harvest (day 4 or 5) than was expected if the biotransformation was completed. The lower than maximal yields of genistein could be the result of degradation of genistein during the fermentation, or the incomplete bioconversion of *genistin* to form genistein, or chemical degradation. So far we have shown that chemical instability of genistein is not the reason. When genistein was spiked into soybean-based fermentation media and subjected to the standard fermentation conditions of shaking and incubation at 30 °C for 5 days, the material was recovered in full at the end of the 5-day period (Table 1). Biotransformation is our favored hypothesis to explain the disappearance of genistein. Others who have worked in this area with closely related compounds have shown that microbial biotransformations of isoflavonones and flavanones are very common among bacteria and fungi (Ibrahim and Abul-Hajj 1990).

Genistein production by *Sac. erythraea*

During this work, a strain commonly used in our laboratory, *Sac. erythraea* (ATCC 11635), was included as a control in our fermentations. Because there are no previous reports of genistein or isoflavone being produced by *Sac. erythraea*, it was expected that this strain would serve as a negative control. Unexpectedly, *Sac. erythraea* was also found to be capable of producing genistein in soybean-based growth media (Fig. 3B). The amount of genistein found at the end of the fermentation was about half the level seen for *S. roseolus*. The simplest explanation for genistein production at these levels is bioconversion. Consistent with this explanation, *Sac. erythraea* culture broth tested positive for

Table 1 Stability of genistein added to uninoculated shake flask fermentations. Uninoculated shake flasks containing the standard Soybean-based fermentation medium were spiked with genistein. Unspiked flasks contained the same growth medium without any genistein added. Duplicate flasks (uninoculated) were incubated under standard fermentation conditions, (30 °C, 5 days). Flasks were weighed before and after the incubation period, and the loss in weight due to evaporation of the medium was compensated through the addition of sterile water at day 5 prior to sampling. Then 1-ml samples were taken from both day-0 and day-5 flasks and then extracted with equal volumes of ethyl acetate. Solvent extracts were separated from the aqueous broth by centrifugation and 10 μ l was injected for HPLC analysis to determine the concentration of genistein at the two time points

Sample		Genistein (μ g/ml)
Unspiked	Day 0	0.83
Unspiked	Day 5	0.69
Spiked	Day 0	22.07
Spiked	Day 5	22.34

β -glucosidase activity using a fluorescent substrate (4-methylumbelliferyl- β -D-glucoside) according to procedure modeled after that of Reinhold-Hurek et al. (1993). One other Actinomycete, *Streptomyces griseus*, has been more intensively studied, and shown to produce genistein from genistin by biotransformation (Konig et al. 1977; Sariaslani and Kunz 1986). Furthermore, our laboratory now has preliminary data indicating that the ability to produce genistein in soybean media is present in other Actinomycetes as well (unpublished results).

To show that the material was biologically active genistein, a portion of the HPLC-purified material (peak 2, Fig. 3B) was tested in the topoisomerase II plasmid-linearization assay (Fig. 5). Genistein has previously been shown to be a potent inducer of topoisomerase-II-dependent DNA cleavage (Constantinou et al. 1995; Yamashita et al. 1990) and the plasmid-linearization assay results show that the HPLC-purified material had the expected topoisomerase II poison activity.

Because *Sac. erythraea* is used in existing large-scale industrial processes for the production of erythromycin and is invariably grown in a soybean-based growth media, these results imply that the erythromycin fermentation might be a rich source of genistein and other isoflavones. At this point, our interests shifted toward investigating this possibility further.

Since in our experiments both genistein and erythromycin were produced, we sought to determine the fate of genistein during the erythromycin purification pro-

cess. An analysis was performed using an aqueous solution of the authentic standards and the movement of genistein and erythromycin was followed. We used the formerly patented process for erythromycin production and recovery (Bunch and McGuire 1953) on a laboratory scale to simulate the industrial situation. In the first step, the fermentation broth was adjusted to pH 9.5 and extracted with a lesser volume of organic solvent such as ethyl acetate. The results of this extraction (Fig. 6, lane 2) show that both genistein and erythromycin were efficiently co-extracted and concentrated at this step. In the second step the solvent fraction was back-extracted with acidified water. At this point, erythromycin, which is charged at low pH, moved back into the aqueous fraction (Fig. 6, lane 4); however, genistein, which is uncharged at low pH, stayed in the solvent fraction (Fig. 6, lane 5). Therefore, genistein and erythromycin were efficiently co-purified during the solvent extraction of the fermentation broth and then they were efficiently separated from one another during the second step. According to these results, genistein would be expected to appear in the solvent waste stream of the industrial erythromycin process.

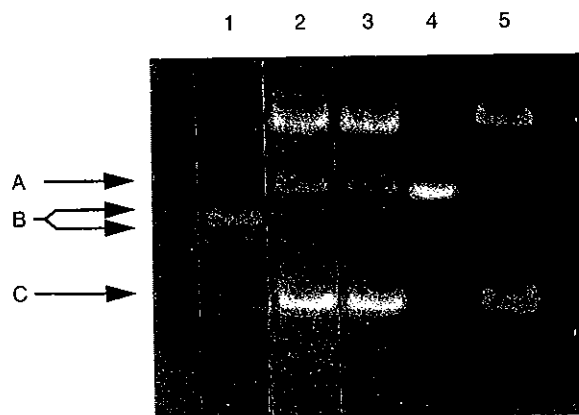


Fig. 5 Topoisomerase plasmid linearization assay. The photo shows a 1% agarose gel run in $1 \times$ TAE buffer, stained with ethidium bromide following electrophoresis. *Lane 1* shows the result of the incubation of topoisomerase II Human p170 form with supercoiled DNA in the absence of genistein, note the presence of the relaxed DNA forms, *arrows B*. *Lane 2* shows the result of the incubation of supercoiled pYRG DNA with topoisomerase II, Human p170 form, in the presence of HPLC-purified material from a *Sac. erythraea* fermentation, note that linear DNA form is present in significant quantity (*arrow A*). *Lane 3* shows the result of the incubation of supercoiled pYRG DNA with topoisomerase II, Human p170 form, in the presence of authentic genistein (Indofine, Somerville, N.J., USA), note the presence of the linear form of pYRG DNA (*arrow A*). *Lane 4* shows the position of linear pYRG DNA (*arrow A*). *Lane 5* shows the position of bands which correspond to two different forms of supercoiled pYRG DNA (*arrow C*) and unlabelled band at *top*

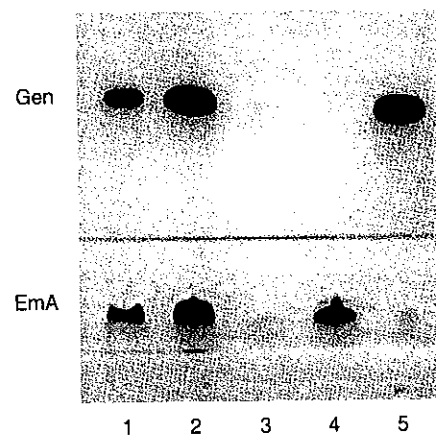


Fig. 6 Solvent extraction of genistein and erythromycin A standards. A mixture of genistein (*Gen*) at 2.5 mg/ml, and erythromycin-A (*EmA*) at 1 mg/ml was prepared in 1 ml of 100% ethanol to which 9 ml of pH 9.5 Tris buffer, 0.05 M, was added (*lane 1*, 4 μ l spotted). One-half volume of ethyl acetate was used to extract this solution of genistein and erythromycin (*lane 2*, 2 μ l spotted of the ethyl acetate fraction; *lane 3*, 2 μ l spotted of the aqueous fraction after extraction). Note that both compounds were completely extracted into the ethyl acetate fraction by this treatment. The solvent fraction containing both genistein and erythromycin was then back-extracted with an equal volume of aqueous sodium succinate buffer pH 5.0, 0.075 M (*lane 4*, 2 μ l spotted of the aqueous phase after the pH 5.0 aqueous extraction; *lane 5*, 2 μ l spotted of the solvent phase after the pH 5.0 aqueous extraction). Note that the erythromycin is effectively back-extracted into the acidified water, but that genistein remains in the solvent phase. Conditions for the development and detection of the TLC plate are given in Materials and methods. The *upper half* of the plate, where genistein would migrate, was photographed under UV illumination. The *lower half* of the plate, where erythromycin-A would migrate, was photographed under natural light following treatment with anisaldehyde to visualize the erythromycin spots

Discussion

In this report we have provided evidence that erythromycin fermentation may be a useful source of large quantities of isoflavones, including genistein. Our results indicate that the genistein produced would be in a highly purified form and could be easily concentrated because it ultimately goes to the solvent waste stream of fermentation operation as described by Bunch and McGuire (1953). Estimating from the large amount of soybeans consumed by the erythromycin fermentation throughout the world, and knowing the amount of genistin in soybeans (Wang and Murphy 1994b), if all of the genistin was completely biotransformed and also completely recovered as genistein, then the quantity of genistein produced would be in the range of tens of metric tonnes.

Our preliminary work presented here, however, indicates that only a small fraction of the genistein produced during the fermentation remains at the end of the fermentation when the erythromycin is harvested. We speculate that additional enzymatic biotransformations (Ibrahim and Abul-Hajj 1990) act on the molecule during the fermentation after its conversion from the glucoside form (genistin). Therefore, in order to optimize this process to make it commercially attractive, additional work needs to be performed to block genistein biotransformation. Fortunately, the producing organism *Sac. erythraea* is well suited for molecular and classical genetic manipulations; if the biotransforming genes were identified and cloned, they could be easily inactivated by targeted gene disruption techniques that are well developed in this organism.

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