IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN: 2278-3008, p-ISSN:2319-7676. Volume 9, Issue 3 Ver. I (May -Jun. 2014), PP 49-60
www.iosrjournals.org

Isolation, screening and characterization of Streptomyces espinosus from rhizospheric soil for Genistein production

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Abstract: *Genistein is a phytoestrogen and belongs to the category of isoflavones. Most of the isoflavones in plants are present in a glycosylated form. The unglycosylated aglycones can be obtained through various means such as treatment with the enzyme β-glucosidase, acid treatment of soybeans followed by solvent extraction, or by chemical synthesis. Acid treatment is a harsh method as concentrated inorganic acids are used. Both enzyme treatment and chemical synthesis are costly. Chemically synthesized genistein or genistein extracted from soyabean currently can cost upto $5,000 a gram. A more economical process consisting of fermentation for in situ production of β-glucosidase to isolate genistein. They are naturally occurring chemical constituents that may interact with estrogen receptors to produce weak estrogenic or anti-estrogenic effects. Because of potential clinical use of genistein as chemopreventive and/or chemotherapeutic agent it is highly important to produce this isoflavone. The present invention provides an alternate process for the large scale production of genistein by Streptomyces espinosus. Fifty three filamentous bacterial isolates were isolated from total of ten soil sample collected from different sites of Mumbai out of which one colony was found to produce Genistein which could be used for further studies and it was termed as Z. On the basis of morphological, physiological, biochemical and molecular characterization the isolated strain Z was confirmed as Streptomyces espinosus and the metabolite produced by Streptomyces espinosus was characterized by HPLC, Mass, NMR and IR spectroscopy and confirmed as genistein.*

Abbreviation: *Streptomyces espinosus, Genistein, NMR, HPLC*

I. Introduction

Genistein was first isolated in 1899. It is one of the several known isoflavones. The main sources of Genistein are soybeans. It acts as a phytoestrogen, antioxidant & anticancer agent. The Genistein which we have isolated is produced by *Steptomyces espinoses*, which has not been reported so far. Genistein is a phytoestrogen (estrogen-like chemical compound present in plants) that is derived from certain plant precursors by human metabolism. They are naturally occurring chemical constituents that may interact with estrogen receptors to produce weak estrogenic or anti-estrogenic effects. They are composed of a wide group of non-steroidal compounds similar in structure and function to human estrogens (Leclerq G. and Heuson J.C., 1979). A conspicuous feature of the chemical structure of phytoestrogens is the presence of a phenolic ring that, with few exceptions, is a prerequisite for binding to the estrogen receptor. For this reason, phytoestrogens can act as weak estrogen agonists, partial agonists, or as antagonists to endogenous estrogens (such as estradiol) and xenoestrogens (including phytoestrogens) with estrogen receptors in both animals and humans. Therefore, working as estrogen, genistein mimics phytoestrogens. It may either have same effects as estrogen or block estrogen's effects. There are three major classes of plant chemical compounds that have estrogen-like actions in the body. They are lignans (enterolactone, enterodiol), isoflavones (genistein, daidzein, biochanin A), and coumestans. The two major chemical classes of phytoestrogens found in people's diets are lignans (enterodiol and enterolactone) and isoflavones (daidzein, genistein, and glycitein). Genistein is an isoflavone. Isoflavone are a class of flavonoids, natural products typically isolated in glycosylated form from plants. The aglycone is the biologically active form that has the most medicinal and commercial interest.

The glycosylated form of genistein is known as genistin. The preparation of genestein must include a step in which the core isoflavone structure (genistein) is separated from the glucose moiety. Genistin, which is found in soyabean, is converted to the biologically active form, genistein, through the action of a beta-glucosidase enzyme. Genistein is reported to be an inhibitor of eukaryotic DNA topoisomerase (topo) I and II. Genistein is used as a chemopreventive agent in animal studies, is required for clinical trials as a dietary supplement; and likely has application as a chemotherapeutic agent when coupled to anti-tumour specific antibodies. Because of potential clinical use of genistein as chemopreventive and/or chemotherapeutic agent it is

highly important to produce this isoflavone and some of its promising structural analogs in larger quantities and to reduce its price. Genistein is currently expensive. Chemically synthesized genistein or genistein extracted from soyabean currently can cost upto $5,000 a gram.

Members of the genus Streptomyces are filamentous Gram-positive soil bacteria with a typical base composition of 72-75 mol% G + C (Enquist & Bradley, 1971; Piepersberg, 1993). Streptomyces undergo complex morphological differentiation including growth of substrate mycelia in the initial phase, followed by development of aerial mycelium and its subsequent conversion to spores (Piepersberg, 1993). Streptomyces possess extensive secondary metabolic pathways leading to the production of a wide array of bioactive compounds including commercially important substances such as antibiotics. The metabolic diversity of these indigenous soil micro-organisms has been widely exploited in industry and agriculture (Mehling A. et al., 1995).

The present invention provides an alternate process for the large scale production of genistein by *Streptomyces espinosus*, the organism that is used in the production of lincomycin. To best knowledge of authors and thorough literature search it is concluded that Genistein produced by *Steptomyces espinosus* is novel and not reported before.

II. Materials and Methods

Isolation and Screening of Genistein producing Actinomycetes

Soil samples were collected aseptically from different sites of Mumbai, India at a depth of 6-12 inches. Soil sample was dried in hot air oven at 50 ^{o}C for 1 hr to minimize the amount of bacteria other than Actinomycetes (Williams et al., 1972; Arunachalam et al., 2010). Standard dilution plate technique was followed for the isolation of Actinomyctes (Kuster and Williams, 1964). 10 gm each of soil sample was added to 90 ml distilled water in a 250ml Erlenmeyer flask under sterile condition and kept in rotary shaker (120rpm) at room temperature for 1 hr. Supernatant was collected and serially diluted to obtain 10^{-4}, 10^{-5} and 10^{-6} dilutions. Each dilution was plated on nutrient agar and Actinomycetes agar medium supplemented with Nalidixic acid. After inoculation of 7-9 days at 27±1^{o}C the actinomycetes colonies were selected, counted and made into pure culture following single spore culture technique. The culture was maintained on yeast malt extract agar by periodic sub-culturing. The pH was adjusted to 7.2± 0.1 before autoclaving the medium at 121^{o}C for 15 min.

Phenotypic characterization of isolated actinomycetes

The isolated strain was morphologically and biochemically characterized according to Bergey's Manual of Determinative Bacteriology by Holt et al. (1994).

DNA isolation for genotypic characterization

Isolation and purification of DNA were carried out according to the method described in Sambrook and Russell, (2001). For the isolation of genomic DNA pure cultures were grown in YM broth till log phase was achieved. 1.5 ml of bacterial culture was centrifuged for 10 min at 8,000 rpm to get bacterial pellets. The supernatant was discarded and the cells were washed with 1 ml cold TE buffer (pH 8.0). 180 µl lysis buffer and 2 µl of RNase were added to the cell pellets. The cell pellets were resuspended by vortex and incubated at 37 ^{o}C for 60 min. Then 25 µl proteinase K and 200 µl AL buffer were added, mixed by vortex for few seconds and incubated at 56 ^{o}C for 30 min. Homogenous mixture was essential for efficient lysis. Then 200 µl of ethanol (96 -100 %) was added to the lysate and mixed thoroughly by vortexing for 15 sec. The sample was added in the DNeasy mini spin column and centrifuged at 8000 rpm for 1 min. The flow was discarding through liquid and the spin column was placed in a new 2.0 ml collection tube. 500 µl of AW1 buffer was added to the spin column and centrifuged at 8,000 rpm for 1 min followed by addition of 500 µl AW2 buffer and then centrifuged at 8,000 rpm for 3 min. The DNA was eluted by adding 200 µl of AE buffer directly into the column without spilling to the sides. It was incubated for 5 min at room temperature and centrifuged at 8,000 rpm for 1 min. The eluted DNA was stored at -20^{o}C till further work. DNA samples that had been subjected to electrophoresis through agarose gels were detected by illumination with 300 nm UV light. The photomicrographs of DNA bands were taken by Gel-documentation system.

The isolated genomic DNA was given to Merck Millipore for actinomycetes identification service.

Culture performance in shake flask

Culture performance in shake flask was studied in the following steps.

Development of lab inoculum

Streptomyces espinosus culture on YM slant was harvested with 5 ml of normal saline and 0.5 ml of suspension inoculated in YMB medium (yeast extract 4.0g/l, malt extract 10.0g/l, D-glucose 4.0g/l) 30 ml/250 ml conical flask, and incubated for 48 ± 24 h at 28^{o}C and 240 rpm or till optimum growth appears**.**

Transfer of Lab inoculum into seed media

Grown lab inoculum (3 %) at the age of 48 h was transferred into the seed flask media (Soluble starch 10 g/l, Peptone bacteriological 10 g/l, glycerol 3.0 g/l, yeast extract 3.0 g/l, malt extract 3.0 g/l). These flasks were incubated at 28^{o}C at 240 rpm up to 36 ± 12 h or till growth appears.

Transfer of seed inoculum into Production media

Different media were selected from the available literature and formulated as per nutritional value (carbon and nitrogen source) (P1 – P12) and tested for the production of Genistein.

The selected grown seed (10%) were transferred to production flask, and incubated at 28^{o}C at 240 rpm for 48 h then temperature was changed to 24.5^{o}C. feeding was done with anyone of the mentioned feeding solutions like 2.0 ml of 50% dextrose (pH 2), 10 % of glycerol (pH 2) and 10 % dextrine white (pH 2.0) to maintain pH 6.5 of production flask. The process parameters pH, PMV%, microscopy analysis was monitored from 72 h to 312 h.

COMPONENTS(g/L)	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
Dextrose	-	-	20	25	20	-	25	50	50	35	25	25
Dextrin White	50	110	-	-	-	-	-	-	-	-	-	-
Soluble Starch	-	-	35	-	-	50	50	-	-	-	35	35
Soya Peptone	-	12	-	-	-	-	5	15	5	-	5	-
Yeast Extract	-	-	-	5	5	5	5	5	5	-	5	-
Malt Extract	-	-	-	10	10	5	10	10	5	-	5	-
Protease Peptone	5	-	-	10	5	5	5	5	5	-	5	-
Soyabean Flour	15	8	30	-	10	-	10	-	-	25	10	15
Cotton Seed Flour	10	10	-	-	5	-	5	-	-	10	5	5
KH_2PO_4	1	2	35	1	1	0.5	1	1	-	2	1	1.5
K_2HPO_4	-	-	3	-	-	-	-	-	-	-	0.75	1
$MgSO_4$	-	-	-	1.5	-	0.5	1	0.5	1	2	1	1
$CaCO_3$	2	2	-	2	2	1	1	1	2.5	2.5	0.5	2
$(NH_4)SO_4$	-	-	2	-	-	-	-	-	-	-	1	1
NaCl	-	-	7	2	2	2	2	1	2	-	2	2
Glycerol	-	8	30	20	-	10	15	25	10	10	-	10
PEG 400	5	12.5	-	10	-	-	-	-	-	15	-	-
Antifoam	-	-	-	-	-	-	-	-	-	-	-	-
Cornsteep Liquor	-	-	-	-	20	-	-	-	50	-	-	25
pH	7	7	7	7	7.5	7	7	7	7.5	7	7	7.5

Trace Elements	(g/l)
$ZnSO_4$	2.0
$MnSO_4$	0.2
$FeSO_4$	10
$CuSO_4$	0.2

Note: **Add 10ml of trace solution to 500ml of each media after adjusting the pH**

Fermentation process for Genistein production

For Genistein production, fermentation process was carried out in the following steps:

Lab Inoculum

35 ml medium was prepared in 250 ml conical flask in duplicate and sterilized at 121^{0}C for 30 minutes.

Media Composition

Medium was prepared having the ingredients as below:

Components	**g/l**
Yeast Extract	4.0
Malt Extract	10.0
D-glucose	4.0
pH	
Actual	6.5
Adjusted	6.8
After Sterilization	6.62

Inoculation and Incubation

Culture suspension from a slant was prepared and inoculated into two conical flasks containing 35 ml sterilized medium under aseptic condition and flask was placed on shaking incubator at 28 ^{0}C, 240 rpm for 48 h.

Maturity of Lab Inoculum

The pH of matured inoculum was observed under range of 6.0 to 6.5 where the packed mass volume was 4.0 to 5.0 %. Filamentous growth with good mycelial network was observed under microscope (100X).

Seed flask (5 Liters)

30 ml of grown inoculum was inoculated into seed flask (5 Liter) with volume of 750 ml media in duplicate and flask was incubated on shaking incubator at 28 ^{0}C, 240 rpm for 48 h.

Media Composition for Seed media-

The ingredients used in the Seed medium are given below.

Components	**g/l**
Soluble starch	10.0
Bacteriological peptone	10.0
Glycerol	3.0
Yeast extract	3.0
Malt extract	3.0
pH	
Actual	6.8
Adjusted to	7.2
After sterilization	7.0

Production Fermenter (22 Litres)

The vessel was physically checked for proper cleaning; availability of utility was also checked before start of batch. The vessel was made ready by doing following process stepwise: Cleaning in Place (CIP), leak test of fermenter, empty sterilization, activation and calibration of pH and DO probe, media preparation and sterilization (working volume 12 litre) were done as described for 22 L production fermenter.

Media Composition for 22 L fermenter-

The ingredients used in the fermentation medium given below.

Production media (Z2)	
Components	**g/l**
Dextrine white	100.0
Soya peptone	10.0
Cotton seed flour	10.0
Glycerol	10.0
PEG 400	12.5
KH_2PO_4	2.0
$CaCO_3$	2.0
Trace solution	20.0 ml
pH	
Actual	6.6
Adjusted to	7.0
After sterilization	6.56

Trace Elements	**(g/l)**
$ZnSO_4$	2.0
$MnSO_4$	0.2
$FeSO_4$	10
$CuSO_4$	0.2

Note: 20ml of trace solution to 1000ml of each media after adjusting the pH

After maturity of seed, 1.2 liter of seed volume was transferred to production vessel. The pH of matured seed was observed to be 7.2 where the packed mass volume was 8 %. The initial parameters were set to air 0.5 vvm and gradually increased to 2.0 vvm. Initially agitation was set to 200 rpm which was raised upto 600 rpm. Back pressure was set to 0.5 kg/cm^2 and increased upto 1.07 kg/cm^2 as process required till the age of 288 h.

Assay, Purification & Characterization of Genistein

HPLC grade methanol, ethyl acetate and acetonitrile were purchased from Merck India Limited, Mumbai, India. Deionized water was prepared using MilliQ plus purification system (Millipore, Bradford, USA). Potassium bromide (FTIR grade), DMSO-D_6, $CDCl_3$-D_1 and D_2O-D_2 were purchased from Merck KGaA, Darmstadt, Germany.

2.2 High performance liquid chromatography

Samples were analyzed on Alliance 2690 HPLC (Waters, Milford, MA, USA) system equipped with 2487 UV detector. A Hypersil BDS C18 column (250 mm x 4.6 mm i.d. 5 µm Thermo scientific, USA) was used for chromatographic separation. The mobile phase consisting of A: water: acetonitrile (70:30, v/v) and B: water, with timed gradient programme T (min)/ B (%): 0/20, 17/70, 22/80, 23/50, 25/20, 30/20 with flow rate of 1.0 ml per minute was used. The injection volume was 20µL and the detector wavelength was fixed at 260 nm.

2.3 Mass spectrometry

The LC-ESI/MS and MS/MS analysis was carried out on LCQ-Advantage (Thermo Finnigan, San Jose, CA, USA) ion trap mass spectrometer. The LC part was consisted of an Agilent 1100 series quaternary gradient pump with a degasser and auto sampler. The chromatographic condition described in section 2.2 has been used for the analysis. The source voltage was kept at 3.0 kV and capillary temperature at 250 °C. Nitrogen was used

as both sheath and auxiliary gas. Mass range was kept at m/z 50-500. MS/MS studies were carried out by maintaining normalized collision energy at 30% with the mass range m/z 50-500.

2.4 Semi-preparative HPLC

The unknown impurity was isolated from crude sample of genistein using Waters Auto-purification system consisting of 2525 binary gradient pump, a 2487 UV detector and 2767 sample manager (Waters, Milford, MA, USA). A Peerless Basic C18 Column (250 mm X 21.2 mm ID, particle size 5µm), USA was used for preparative isolation. The mobile phase consisting of A: water: acetonitrile (70:30, v/v) and B: water, with timed gradient programme T (min)/ B (%): 0/100, 10/100, 11/0, 80/00 with flow rate of 35.0 ml per minute was used. The injection volume was 5.0 mL and the detector wavelength was fixed at 260 nm.

2.5 NMR

The ^{1}H, ^{13}C measurements of synthetically prepared impurity sample were recorded on a AVANCE 400 (Bruker, Fallanden, Switzerland) instrument at 300 K. DEPT spectral editing revealed the presence of methyl and methine groups as positive peaks while the methylenes as negative peaks. The exchangeable protons were identified by D_2O exchange experiment. The phase sensitive double quantum filtered correlation spectroscopy (DQF-COSY), hetronuclear single quantum correlation (HSQC) and nuclear Overhauser effect spectroscopy (NOESY) was also performed using the same instrument. The ^{1}H and ^{13}C chemical shift values were reported on the δ scale in ppm relative to DMSO-d_6 (2.50 ppm) and (39.5 ppm) respectively.

2.6 IR spectroscopy

The IR spectrum of isolated impurity was recorded in the solid state KBr powder dispersion using a Spectrum-One FT-IR spectrometer (PerkinElmer, Beaconsfield, UK).

III. Results

Fifty three filamentous bacterial isolates were isolated from total of ten soil sample collected from different sites of Mumbai out of which one colony was found to produce Genistein which could be used for further studies and it was termed as Z. On YM plates isolate Z colonies were pin-point in size, circular in shape, regular in margin, umbonate in elevation, dark pink in colour with opacity, hard to pick and sporulate with age The isolate Z gives morphological similarity to that of a Streptomyces espinosus with a distinct mycelium as observed through light microscopy. Isolate Z was gram positive, non-motile with Nitrate and sugar fermentation positive while Oxidase, Catalase, Citrate, MR-VP, Starch Hydrolysis, Casein hydrolysis, Fluorescence assay, Indole production was negative as shown in Figure 1 and Table 1.

Figure 1: Genistein producing strain Streptomyces espinosus Z. (A) Growth and colony morphology of S. espinosus on YMA plate; (B) Morphological characterization of *S. espinosus* through light microscopy

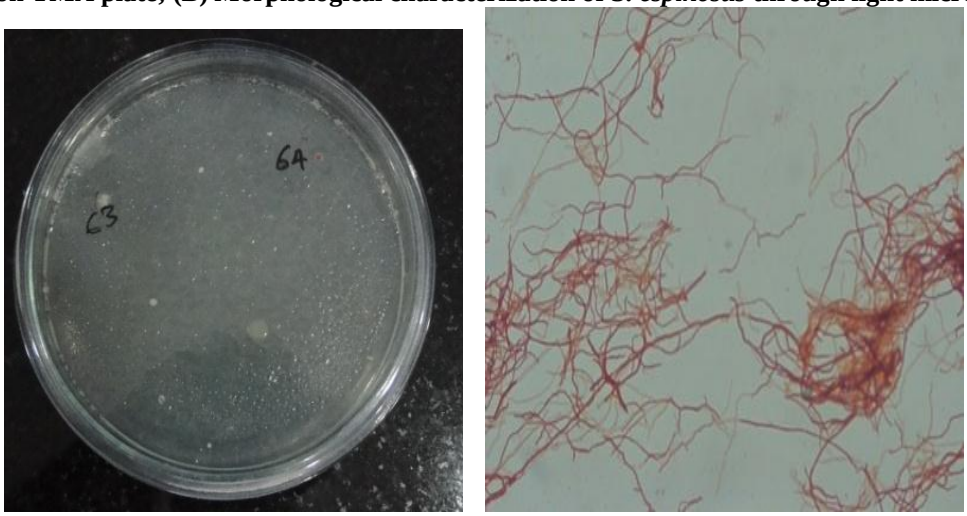


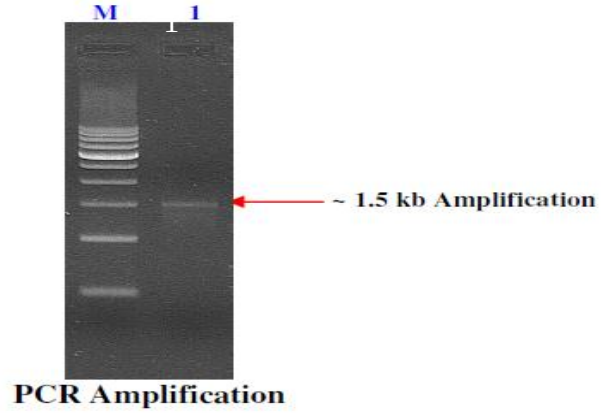
Table 1: Morphological, physiological and biochemical characteristics of actinomycetes isolate Z

S.No.	Characteristics	Actinomycetes isolate Z
1	Soil sample	Garden soil
2	Colony no.	Z
3	Size	Pinpoint
4	Shape	Circular
5	Margin	Regular
6	Elevation	Umbonate
7	Colour	Dark Pink
8	Opacity	Opaque
9	Consistency	Hard to pick and sporulation with age
10	Gram reaction	Positive
11	Microscopic shape	Mycelial growth
12	Motility	Non-motile
13	Oxidase	-
14	Nitrate	+
15	Catalase	-
16	Citrate	-
17	MR-VP	-
18	Starch Hydrolysis	-
19	Casein Hydrolysis	-
20	Fluorescence assay	-
21	Sugar fermentation	+
22	Indole production	-

IV. Molecular characterization

Isolation of genomic DNA and amplification of 16S rRNA gene

Genomic DNA was isolated from *S. espinosus* as shown in Figure. Further, it was used for the amplification and sequencing assessment. The 16S rRNA gene of the isolates was amplified by using universal primers Univ F and Univ R. The amplified products were compared with low range ruler DNA. The size of amplified product was approximately 1500 bp which represented the amplification of nearly full length 16S rRNA gene from the isolates. The size of the PCR product corresponded with the size of the 16S rRNA genes among the isolates (Figure 2).



Abbreviation: M: Marker; 1: Sample

Figure 2: Amplified 16S rDNA gene of S. espinosus.

Characterization of strain on the basis of 16S rDNA sequencing

The strain was identified for their taxonomic position on the basis of phylogenetic analysis of 16S rDNA sequences. The strain Z was identified as Streptomyces espinosus (Figure 3).

Phylogenetic analysis

A neighbour joining dendogram was prepared using 16S rDNA sequences of *Streptomyces espinosus* Z and representative sequences from EMBL/Gen Bank/DDBJ and PDB. Gene sequence was used to carry out BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score first ten sequences were selected the phylogenetic tree was constructed using MEGA 4.

Two major clades were observed in which isolate Z clustered with *Streptomyces espinosus* (X80826) with 97% bootstrap values (Figure). Based on nucleotides homology and phylogenetic analysis the microbe (Sample: DSPORE) was found to be *Streptomyces espinosus* (GenBank Accession Number: X80826). Nearest homolog was found to be Streptomyces *sp.* Durck 47 (GenBank Accession Number: HE664170).

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 603 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

The 16S rDNA sequences of the *Streptomyces espinosus* Z

Aligned Sequence Data: (1468bp)

AGAAGAGTTTGATCATGGTTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCACCTTCGGGT
GGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTTCGGGACAAGCCCTGGAAACGGGGTCTAAT
ACCGGATACGACACCGGGAGGCATCTCCTCGGTGTGGAAAGCTCCGGCGGTGCAGGATGGGCCCGCGGCCTATCAGCTGGT
TGGTGAGGTAACGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGC
CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGA
CGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGCGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCCTGTCGC
GTCGGTTGTGAAAGCCCGGGGCTCAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAAT
TCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTG
AGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCGG
CATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
ATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATAC
GCCGGAAAACCCTGGAGACAGGGTCCCCCTTGTGGCCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCCGTGTTGCCAGCAGGCCCTTCGGGGTGCTGGGGACTCACGGGA
GACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGTTA
CAATGGCCGGTACAATGAGCTGCGATGCCGCGAGGCGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGC
AACTTGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCACTGCTCCGGTGAATACGTTCCCGGGCCTTGTACAC
ACCGCCCGTCCCGTGCTAAAGTCGGTAACACCCGAAGCCGGGGCCCAACCCCTCGCGGGAGGAGCCTAGTATATCTCCAGC
ACCTCCATAC

Alignment view using combination of NCBI GenBank and RDP database:

Alignment View	ID	Alignment results	Sequence Description
	Sample Z	1.00	Studied Sample
	HE664170	0.97	*Streptomyces sp. Durck47 st.Durck47*
	X80826	0.97	*Streptomyces espinosus st.NRRL 5729*
	AB184662	0.95	*Streptomyces ghanaensis st.NBRC 15414*
	JX204833	0.96	*Streptomyces caelestis st.AW9-9C*
	GU045531	0.96	*Streptomyces sp.SXY48*
	HQ850413	0.95	*Streptomyces cellulosae st.S44*
	EF371427	0.95	*Streptomyces vinaceus st.3078*
	EF626594	0.95	*Streptomyces brasiliensis st.NRRL B-3327*
	GU433227	0.96	*Streptomyces calvus st.ABRIINW 673*
	AY999902	0.95	*Streptomyces asterosporus st.NRRL B-24328*

Distance Matrix based on Nucleotide Sequence Homology (Using Kimura-2 Parameter)

Distance Matrix based on Nucleotide Sequence Homology (Using Kimura-2 Parameter):

	1	2	3	4	5	6	7	8	9	10	11
1 Z	0.000	0.062	0.105	0.101	0.117	0.123	0.095	0.095	0.102	0.115	0.114
2 HE664170	0.062	0.000	0.056	0.041	0.074	0.080	0.054	0.051	0.059	0.072	0.071
3 X80826	0.105	0.056	0.000	0.064	0.054	0.050	0.077	0.074	0.057	0.038	0.068
4 AB184662	0.101	0.041	0.064	0.000	0.090	0.095	0.082	0.079	0.072	0.084	0.085
5 JX204833	0.117	0.074	0.054	0.090	0.000	0.014	0.033	0.030	0.040	0.033	0.047
6 GU045531	0.123	0.080	0.050	0.095	0.014	0.000	0.040	0.038	0.045	0.029	0.054
7 HQ850413	0.095	0.054	0.077	0.082	0.033	0.040	0.000	0.004	0.044	0.056	0.057
8 EF371427	0.095	0.051	0.074	0.079	0.030	0.038	0.004	0.000	0.041	0.053	0.055
9 EF626594	0.102	0.059	0.057	0.072	0.040	0.045	0.044	0.041	0.000	0.035	0.032
10 GU433227	0.115	0.072	0.038	0.084	0.033	0.029	0.056	0.053	0.035	0.000	0.033
11 AY999902	0.114	0.071	0.068	0.085	0.047	0.054	0.057	0.055	0.032	0.033	0.000

Table indicates nucleotide similarity (above diagonal) and distance (below diagonal) identities between the studied sample '**Sample-Z**' and ten other closest homologous microbe.

Phylogenetic Tree made using Neighbor Joining method:

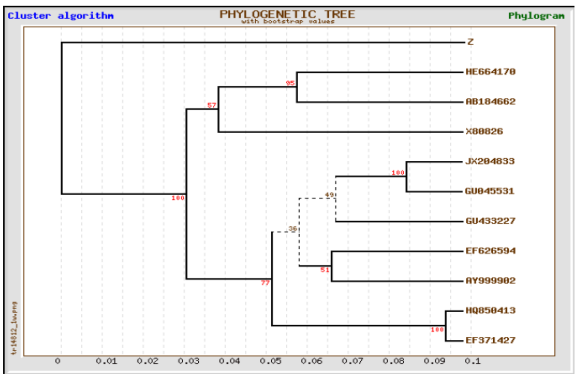


Figure 3: Phylogenetic relationship between Streptomyces espinosus Z and representative Streptomyces sp. based on partial 16S rDNA sequences. The tree was constructed by maximum parsimony method based on 16S rDNA sequences. Accession number of strains is shown in dendogram. Number above the branches is bootstrap values based on 1000 replicates with fast step-wise addition.

Culture performance in shake flask

S. espinosus was inoculated in lab inoculum, then 3 % of 48 h grown lab inoculum was transferred into seed medium. Later 10 % of matured seed was transferred to different production medium for Genistein production. In process parameters, i.e. pH, PMV was measured at different interval as shown in Table 2.

Table 2: Analysis of process parameter in P1 to P12 Production medium

Production	Time (h)	pH	PMV (%)	Production	Time (h)	pH	PMV (%)
	72	6.43	-		72	6.79	-
P1	120	6.97	-	**P9**	120	5.98	-
	168	5.34	15		168	7.44	25
	216	7.73	30		216	7.28	25
	264	6.04	25		264	7.36	25
	312	7.00	45		312	7.43	30
	72	6.17	-		72	6.16	-
	120	5.64	-		120	4.98	-
P2	168	6.45	25	**P10**	168	4.88	15
	216	6.62	30		216	4.77	15
	264	6.7	30		264	5.84	25
	312	6.45	55		312	8.28	50
	72	5.25	-		72	5.62	-
	120	4.68	-		120	5.15	-
P3	168	4.61	30	**P11**	168	5.62	20
	216	4.63	10		216	5.8	20
	264	5.3	30		264	6.98	20
	312	4.55	25		312	7.77	25
	72	6.57	-		72	5.78	-
	120	7.22	-		120	5.34	-
P4	168	7.2	20	**P12**	168	5.94	20
	216	7.97	25		216	6.42	30
	264	7.48	20		264	6.28	25
	312	6.03	30		312	4.93	20
	72	6.66	-				
P5	120	6.17	-				
	168	7.5	35				
	216	8.17	35				
	264	7.74	40				
	312	7.5	43				
	72	7.09	-				
	120	7.1	-				
P6	168	6.4	15				
	216	7.0	15				
	264	7.63	20				
	312	7.63	15				
	72	5.75	-				
	120	5.09	-				
P7	168	5.52	25				

	216	6.03	20
	264	5.7	10
	312	7.52	40
	72	6.7	-
	120	4.97	-
P8	168	4.63	15
	216	4.55	10
	264	4.24	10
	312	4.31	10

V. Fermentation process for Genistein production

Maturity of Lab Inoculum

At 48 h, pH and PMV of lab inoculum was 6.6 and 3 %, respectively. Filamentous growth with good mycelial network was observed through microcopy without any contamination. 35 ml of said inoculum was inoculated into seed flask with volume of 750 ml seed media.

Seed inoculum

At 48 h, pH and PMV of seed inoculum was 7.14 and 8 %, respectively. Microscopic observations looked like filamentous growth with good mycelia network. 1.5 liter of grown seed was transferred into production fermenter with volume of 12 liter.

Production Fermenter

In process parameters (such as pH, PMV, DO, air, BP, RPM) were measured in production medium at different intervals (Table 3). pH was maintained by feeding 10 % dextrine white of pH 2.0. At the start of fermentation process following parameters of medium were taken into account, such as at 0 h temperature 28.1^{o}C, pH 6.35, PMV 7 %. DO, air, BP and speed were recorded as 100 %, 17 LPM, 0.60 kg/cm^2, 200 RPM, respectively. The results of HPLC assays showed that the organism started producing Genistein from 72 h. At 72 h, RPM increased to 600 and air by 48 LPM. At 96 h, pH, PMV, DO, BP were measured as 6.35, 20 %, 52%, 0.63 kg/cm^2. Air remained the same 48 LPM and RPM to 600. At 120 h, DO came down to 39.6% and air to 45 LPM. At 144 h, air decreased to 28 LPM, DO was 35.1%. At 160 h, parameters like DO 27.2 %, air 28 LPM and 500 RPM were set. At 203 h, DO was 19.8 %, air and RPM were 28 LPM, 500 respectively. The packed mycelium volume gradually increases up to 26 %. pH 6.5-7.0 was maintained by feeding of 10% dextrine white of 2.0 pH. Production batch was ran at 28°C upto 48 h and then shift to 25°C temperature. Batch was started with 200 RPM and 17 liter/minute air (i.e. 0.9 VVM). Back pressure rose up to 1.03 kg/cm^2 (Table 3 and 4).

Table 3: **Analysis of process parameter in fermenter production medium**

Age (Hrs)	Temp (°C)	pH	PMV (%)	Air (LPM)	Speed (RPM)	B.P (Kg/cm2)	D.O (%)	Vol. (liters)
0	28.1	6.35	07	17	200	0.60	100	34.5
04	28.1	6.34	07	17	200	0.62	100	-
08	27.9	6.34	07	17	200	0.58	100	-
11	28.1	6.38	08	17	250	0.65	100	-
16	28.1	6.18	08	17	250	0.65	100	-
20	28.0	6.11	11	17	300	0.61	100	-
24	28.0	6.01	12	17	350	0.63	100	-
32	28.0	5.74	16	17	400	0.63	100	-
40	28.0	5.64	20	17	400	0.56	100	-
48	25.0	5.62	17	17	500	0.62	100	-
56	25.0	5.86	17	25	550	0.63	95	32
64	25.2	5.96	17	25	550	0.61	82.8	-
68	25.1	6.02	20	32	600	0.53	77.8	-
72	25.0	6.05	19	48	600	0.59	73.6	-
80	25.0	6.10	19	48	600	0.62	65.9	30
88	24.8	6.38	19	48	600	0.65	61.3	-
96	25.0	6.35	20	48	600	0.63	52.1	-
104	25.0	6.66	20	60	600	0.62	47.4	-
112	25.0	6.92	21	60	600	0.64	46.8	-

116	25.0	6.99	20	60	600	0.65	42.4	-
120	25.0	7.03	18	45	600	0.80	39.6	-
124	25.0	6.93	17	45	600	1.05	37.6	-
128	25.0	7.10	20	45	600	1.02	40.4	-
136	24.9	6.96	23	45	600	1.09	36.7	-
140	25.0	7.14	25	45	600	1.0	34.8	-
144	25.0	7.10	23	28	500	1.02	40.1	-
148	25.0	7.06	22	28	500	1.02	45.0	-
152	25.0	7.02	21	28	500	1.06	48.2	-
160	25.0	7.08	21	28	500	1.03	52.0	-
176	25.0	7.10	21	28	500	1.05	55.0	-
200	24.9	7.15	21	28	500	1.05	60.2	-
203	25.1	7.25	26	28	500	1.03	62.3	-
210	25.0	7.40	28	28	500	1.03	63.0	-
220	25.2	7.49	30	28	500	1.02	65.2	24

Temperature (°C**):** 0 – 42hrs. = 28, 43 to 203 hrs= 25.
Agitation (RPM): 0 – 8 Hrs. =200, 9 - 18 Hrs. =250, 19 -23 Hrs. =300, 24 -31 Hrs. =350, 32 -40 Hrs. =400, 41 – 43 hrs=450, 44 – 55 hrs=500, 56 -67=550, 68 – 143= 600, 144 – 203 hrs= 500.
Air (LPM): 0 – 48 Hrs. =17, 49 - 64= 25, 65 -71 Hrs = 32, 72 – 96 = 48, 97 – 116 = 60, 117 – 140 hrs= 45, 141 – 203 hrs = 28.

Back Pressure (Kg/cm^2): 0 - 120 Hrs. = 0.60, 121 – 203 hrs= 1.0.

Feeding detail:

Age (Hrs)	Feeding Solution	Feeding Volume (Liters)
113	10% Dextrine white (pH 2.0)	0.5
129	10% Dextrine white (pH 2.0)	0.5

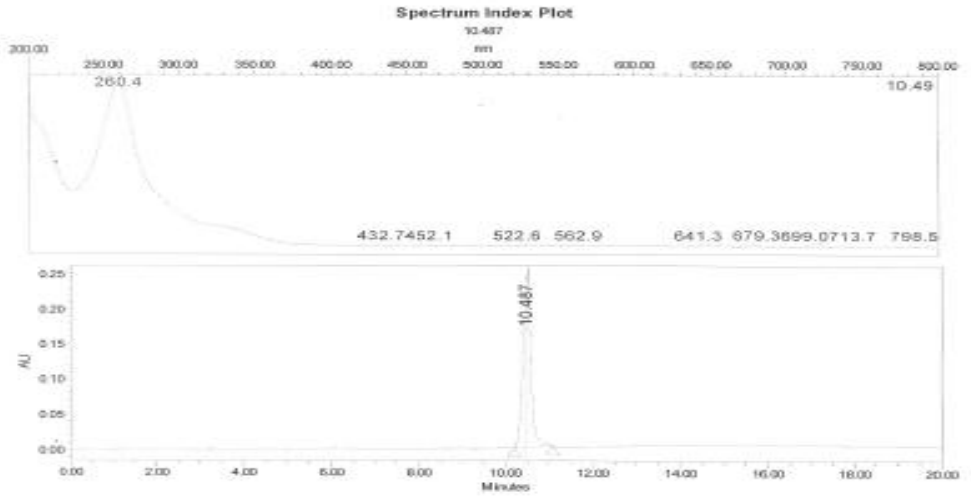
Table 4: Analysis of process parameter in fermenter production medium

Age (Hrs.)	Temp. °C	pH	PMV (%)	TS (g/l)	RS (g/l)	PO_4 (g/l)	NH_3N (g/l)	D.O. (%)	Air (LPM)	B.P. (Kg/cm^2)	Speed (RPM)	Temp. °C (20°C =0)	Speed (rpm/100)	Air (VVM)
0	28.1	6.35	7	75.32	14.25	0.056	0.418	100.0	17	0.60	200	8.10	2.00	0.50
8	27.9	6.34	7	70.84	20.57	0.055	0.339	100.0	17	0.58	200	7.90	2.00	0.50
32	28.0	5.74	16	66.11	26.33	0.046	0.258	100.0	17	0.63	400	8.00	4.00	0.50
56	25.0	5.86	17	59.36	30.38	0.050	0.229	96.2	25	0.62	500	5.00	5.00	0.75
80	25.0	6.10	19	49.20	34.11	0.048	0.298	65.5	48	0.62	600	5.00	6.00	1.50
104	25.0	6.66	20	38.23	41.36	0.046	0.280	47.4	60	0.62	600	5.00	6.00	2.00
128	25.0	7.10	20	35.52	50.18	0.031	0.223	40.4	45	1.02	600	5.00	6.00	1.50
152	25.0	7.02	21	30.35	43.66	0.034	0.279	48.2	28	1.06	500	5.00	5.00	1.00
176	25.0	7.10	21	29.95	34.58	0.031	0.249	55.0	28	1.05	500	5.00	5.00	1.00
203	25.1	7.25	26	26.17	29.33	0.029	0.269	62.3	28	1.03	500	5.10	5.00	1.00
210	25.0	7.40	28	20.56	20.23	0.028	0.260	63.0	28	1.03	500	5.10	5.00	1.00
220	25.2	7.49	30	15.59	10.36	0.023	0.251	65.2	28	1.02	500	5.20	5.00	1.00

Assay, Purification & Characterization of Genistein

The material obtained from production media was centrifuged at 4700 rpm for 10 minutes at 25 °C. The settled cake from this process was then extracted with ethyl acetate. 2.5 mL of this extract was diluted to 25 mL with methanol. The HPLC chromatograph showed the major peak at 10.48 RT (see the fig 4.) which was chemical entity of interest. The same sample was then subjected to LC-MS/MS analysis. The MS spectral data of peak at 10.41 min gave $[M+H]^+$ ion peak at m/z 271 and MS/MS ion peaks at m/z 253, 243, 215 and 153. Based on the mass and HPLC data it was not possible to characterize the major peak. Semi-preparative HPLC was employed for isolation of major peak of interest.

Figure 4: HPLC Graph with PDA Spectra



The obtained material was lyophilized and with purity by HPLC greater than 99 %. The ^{1}H NMR of isolated material revealed the presence of 7 aromatic protons with 3 exchangeable protons (confirmed by D_2O exchange). ^{13}C and DEPT-135 NMR spectra data revealed presence of total fifteen carbon atoms with seven methylene and eight quaternary groups.

The proposed structure of 5,7-Dihydroxy-3-(4-hydroxyphenyl)chromen-4-one i.e Genistein was also supported by FTIR analysis. The confirmation of this compound was then carried out by co-injecting the commercially available Genistein reference material.

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