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ORIGINAL ARTICLE

Unravelling the cellulolytic and hemicellulolytic potential of *Streptomyces griseoincarnatus* SSPJ4 from semi-arid agricultural region: genome analysis and saccharification studies

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ABSTRACT

Background and Objectives: Second-generation bioethanol, a sustainable and environmentally friendly alternative to fossil fuels, can significantly contribute to the economy. However, saccharification accounts for approximately 20-25% of total bioethanol production costs, which can be reduced by using the improved enzyme cocktails. Therefore, the present study aimed to isolate and characterize potent cellulolytic *Streptomyces* strains capable of enhancing the hydrolytic conversion of lignocellulosic biomass.

Materials and Methods: Degraded wood and soil samples were collected and enriched with paddy straw. The isolates were obtained using standard methods and characterized by 16S rRNA sequencing. The best isolate was subjected to whole-genome sequencing to identify hydrolytic genes involved in agro-waste degradation. Saccharification assays were performed using crude culture filtrate in combination with the commercial cellulase cocktail Celluclast® to evaluate sugar release from alkali-pretreated paddy straw.

Results: Among the 38 isolates obtained, five were identified as potential cellulolytic *Streptomyces* strains based on 16S rRNA gene sequencing: *Streptomyces tunisiensis* SSPJ1, *Streptomyces griseoincarnatus* SSPJ4, *Streptomyces werraensis* SSPJ14, *Streptomyces ardesiacus* SSPJ32, and *Streptomyces tendae* SSPJ48. *S. griseoincarnatus* SSPJ4 emerged as the most promising isolate, demonstrating significantly higher enzyme activities. *S. griseoincarnatus* SSPJ4 was subjected to whole-genome sequencing using the GALAXY tool for assembly and annotation, with an assembled genome of 7.3 Mb, 72.38% G+C content, and 6678 genes. Biosynthetic gene clusters (23) were identified using antiSMASH, and 226 proteins were annotated with CAZy domains, of which 121 belonged to the glycoside hydrolase (GH) family. Supplementation of Celluclast® with the crude enzyme preparation from *S. griseoincarnatus* SSPJ4 enhanced the saccharification of alkali-pretreated paddy straw, resulting in a 1.55-fold higher sugar yield compared with Celluclast® alone.

Conclusion: The findings demonstrate the considerable potential of *S. griseoincarnatus* SSPJ4 as a source of accessory lignocellulolytic enzymes for improving biomass saccharification. Its enzyme repertoire could be exploited to develop cost-effective and efficient enzymatic formulations for second-generation bioethanol production.

Keywords: Whole-genome sequencing; *Streptomyces*; Saccharification; Biofuel; Bioethanol

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INTRODUCTION

The quest for sustainable energy alternatives has become urgent due to pressing environmental concerns and the impending depletion of fossil fuel reserves. Unlike traditional energy sources such as fossil fuels, biofuels have emerged as an alternative to reduce greenhouse gas emissions and dependence on finite energy sources (1). Biofuels can be derived from photosynthetic microorganisms, feedstocks, and agro-waste materials. Lignocellulosic biomass, a renewable feedstock, consists of cellulose, hemicellulose, and lignin, which can be hydrolysed into monomeric sugars by enzymatic action. However, the high cost of saccharification enzymes hampers the overall economy of bioethanol production from lignocellulosic biomass (2). The complete degradation of lignocellulosic biomass requires the action of different enzymes. Commercial cellulases contain a cocktail of cellulases, hemicellulases, and lytic polysaccharide monooxygenases (LPMOs) that act synergistically on lignocellulosic biomass (3).

Hemicellulose, a major component of plant cell walls, contains arabinoxylan and arabinan as key polymers, which require specific enzymes, such as endo-arabinase and arabinofuranosidase, for complete hydrolysis. Accessory enzymes remove the side chain and improve substrate accessibility through efficient hydrolysis (4), thereby reducing bioethanol production costs. Therefore, there has been increased interest in highly efficient enzyme mining and further understanding of enzymatic degradation mechanisms (5). Optimization, appropriate enzyme ratios, and protein engineering can increase saccharification yields (6).

Lignocellulolytic enzymes can be obtained from fungi, bacteria, and actinomycetes, with fungi being the most abundant source. Fungi are used for their stable enzymes and higher enzyme titer. Actinomycetes are an interesting group of Gram-positive filamentous bacteria known for producing secondary metabolites, including enzymes and antibiotics. Actinomycetes, particularly the genus *Streptomyces*, are prolific producers of hydrolytic enzymes that catalyze the breakdown of complex polysaccharides. *Streptomyces* species are known to be good candidates for cellulase production, as they can efficiently degrade cellulose and other components of plant biomass (7-11).

Conventional screening methods based solely on enzyme activity often fail to identify the full hydrolytic potential of a microorganism. Genome mining combined with whole-genome analysis has emerged as a powerful tool to discover novel metabolites and gene clusters and unlock the unrevealed potential of *Streptomyces* (12). Whole-genome analysis has extensively explored novel genes and biosynthetic pathways for producing bioactive compounds. It enables the comprehensive identification of CAZymes gene families, including glycohydrolases (GH), such as GH43, GH51, and GH62, which are responsible for the degradation of arabinan.

Streptomyces griseoincarnatus has been isolated from diverse sources, including marine sediments, soil, and cold deserts, and several strains have undergone whole-genome sequencing and preliminary CAZyme annotation (13-15). However, no previous studies have reported its isolation from semi-arid agricultural regions coupled with comprehensive CAZy-level annotation targeting accessory hemicellulolytic genes critical for paddy straw degradation. The present study was undertaken to characterise the newly isolated potential hyper-hemicellulolytic *S. griseoincarnatus* SSPJ4 from a semi-arid region using whole-genome analysis and to utilise its crude enzyme for potential application in paddy straw hydrolysis.

MATERIALS AND METHODS

Isolation, screening, and characterisation of cellulase-producing actinomycetes. Degraded wood and soil samples were collected from different sites at the Central University of Haryana, Mahendergarh, India (28.3513°N, 76.1337°E). The samples were enriched with paddy straw in a ratio of 1:5 with Reese's Minimal Media (RMM). The isolation was done on RMM enriched with 1% (w/v) carboxymethyl cellulose for 3-5 days at 30 ± 2°C. Morphologically different colonies were streaked on starch casein agar (pH 9.0) and maintained for further use.

Screening of isolated actinomycetes: quantitative screening. The isolated actinomycetes were grown on RMM-enriched 1% (w/v) carboxymethyl cellulose (CMC-RMM) and birchwood xylan (BWX-RMM) media and incubated at 30 ± 2°C for 48 h. The plates were flooded with Congo Red solution (0.1% w/v) and washed with 1 M NaCl solution. A clear zone around the colony indicated the hydrolysis

of polysaccharides to monosaccharides and the presence of the cellulase. The cellulose-degrading ability of isolates was measured by calculating the hydrolysing index (HI):

$$eq.\ 1\text{-}\ Hydrolyzing\ index\ (HI)=\frac{Diameter\ of\ zone-Diameter\ of\ colony}{Diameter\ of\ colony} \quad \text{(Eq. 1)}$$

Molecular characterisation. The five isolates showing higher hydrolysing index (HI) on both CMC-RMM and BWX-RMM were selected. The isolates were cultivated on Nutrient Broth (HiMedia) for 24 h at 30 ± 2°C under constant shaking at 250 rpm. The cells were recovered and used for genomic DNA (gDNA) isolation using the Zymo Research™ Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research Corp., Irvine, CA, USA, Catalogue # D6005) following the manufacturer's instructions. The universal primers 27F and 1492R were used to amplify the 16S rRNA gene from gDNA. The purified PCR products were sequenced using the same primers at Biologia Research India (BRI, Karnal, India). The partial DNA sequences analysis of 16S rRNA was performed by comparing it with the Ezbiocloud database (https://www.ezbiocloud.net/identify).

Enzyme activities. The selected potential isolates were subjected to quantitative screening under submerged fermentation (SmF) in RMM with 1% paddy straw as a carbon source at 30 ± 2°C for 96 h. Exoglucanase, endo-glucanase, xylanase, and endo-arabinase activities were quantified using the DNSA test (16, 17). One unit enzyme activity is defined as the amount of enzyme producing 1 µmol of product in 1 min (U/ml) under experimental conditions.

Whole-genome Paired-End library preparation and sequencing. High-molecular-weight shear-free DNA was isolated. The quality and quantity of the DNA were determined using a QIAxpert high-speed microfluidic UV/VIS spectrophotometer, and the DNA sample had a concentration of 392 ng/µL with a 260/280 ratio of 1.92 and a 260/230 ratio of 1.53, indicating high purity and concentration.

For shotgun library preparation, 0.2 µg of (gDNA) was used as the starting material. DNA was sheared via sonication to generate fragments averaging 350 bp in length. Following fragmentation, the DNA underwent end-repair, A-tailing, and ligation with complete Illumina sequencing adapters. The resulting library underwent size selection and PCR enrichment, after which the amplified products were cleaned using AMPure XP beads (Beverly, USA). Library integrity was subsequently evaluated using an Agilent 5400 instrument (Agilent, USA). Libraries that met the quality thresholds were combined and loaded onto a NovaSeq Illumina platform for paired-end sequencing. Raw sequence reads underwent base calling, with the output stored in FASTQ format containing both nucleotide sequences and per-base quality scores (18). Before downstream analysis and annotation, reads harboring adapter sequences, low-quality bases, or ambiguous nucleotides (N) were filtered out.

Genome assembly and sequence annotation. Assembly and annotation were performed using the web-based server GALAXY (https://usegalaxy.org). Raw paired-end reads were assembled using GALAXY SPAdes, a web-based short-read assembler, as described previously (19). The raw reads were assembled using the recommended program for Illumina sequencing reads. The output after assembly contained contigs and scaffolds of the genome. Genome annotation was performed using GALAXY PROKKA (20). Putative biosynthetic gene clusters (BGCs) were identified using the antiSMASH server (21), and Carbohydrate-Active Enzyme (CAZyme) annotation was performed using the dbCAN meta server (https://bcb.unl.edu/dbCAN2/blast.php). These web-based platforms were selected for their standardized workflows, user-friendly interfaces, and integration of multiple annotation tools, which enable rapid and reproducible genome analysis without requiring high-performance computing infrastructure.

Phylogenomic analysis of *Streptomyces griseoincarnatus* SSPJ4. From the whole-genome sequence, six housekeeping genes, viz., *16S rRNA*, *atpD*, *gyrB*, *recA*, *rpoB*, and *trpB*, were mined and subjected to multilocus sequence analysis (22) using the Galaxy platform (https://usegalaxy.org). Subsequently, taxonomic classification was confirmed using the Type Strain Genome Server (TYGS) (http://tygs.dsmz.de), estimation of average nucleotide identity (ANI), and digital DNA-DNA hybridization with the nearest phylogenetic neighbour.

Submerged fermentation for enzyme production using different biomasses. The procured cellulosic substrates, including wheat bran (WB), paddy straw (PS), pearl millet (PM), and sugarcane bagasse

(SCB), were selected for submerged fermentation. The substrates were ground and subjected to mild oxidative pretreatment with hydrogen peroxide (H_2O_2), except for wheat bran. Fermentation was carried out in 250 mL Erlenmeyer flasks containing 100 mL of RMM medium with 1% substrate (w/v) and 4% (v/v) primary inoculum of *S. griseoincarnatus* SSPJ4. The cultures were incubated on a rotary shaker (120 rpm) at 30 ± 2°C for 15 days. Samples were collected on days 2, 5, 7, 10, and 15. The samples were filtered through filter paper to extract the crude enzymes. Cellulase (FPase and endoglucanase) (16) and hemicellulase (xylanase and arabinase) (17) activities were assayed.

Saccharification for bioethanol production. The saccharification efficiency of the crude enzyme was tested using alkali-pretreated paddy straw, as described (18). Enzymatic hydrolysis was performed in three sets: Celluclast® 15 FPU, 10 U/mL crude enzyme from *S. griseoincarnatus* SSPJ4, and enzymatic cocktail of Celluclast® (15 FPU) and 10 U/mL crude enzyme from *S. griseoincarnatus* SSPJ4. Saccharification was performed at 50°C for 72 h with 0.01% sodium azide at 2% substrate loading (dry weight basis). Samples were withdrawn periodically and analyzed for sugar concentration using the DNSA method. The saccharification percentage was calculated using the following formula:

$$\% \text{ Saccharification} = \frac{Reducing\ sugar\left(\frac{mg}{mL}\right) \times 0.9}{Initial\ substrate\ concentration\ \left(\frac{mg}{mL}\right)} \times 100 \quad \text{[Eq. 2]}$$

Data analysis. All experiments were performed in three biological replicates (n = 3), each with technical triplicates. The results are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA with GraphPad Prism software. A post-hoc test was applied to evaluate the significance of the factors. Differences were considered significant at $p < 0.05$.

RESULTS

Isolation, screening, and characterization of cellulolytic microbes. Thirty-eight morphologically distinct spore-forming actinomycetes that developed clear zones on carboxymethyl cellulose (CMC) and birchwood xylan (BWX) agar plates were isolated, indicating their hydrolytic potential. The top five isolates exhibiting a hydrolysis index (HI) >15 mm on both substrates were selected for further analysis. These isolates were identified using 16S rRNA sequencing and matched with the EzBioCloud database (https://www.ezbiocloud.net/identify). The identified strains included *Streptomyces tunisiensis* SSPJ1 (accession no. PV012471), *Streptomyces griseoincarnatus* SSPJ4 (accession no. PV012472), *Streptomyces werraensis* SSPJ14 (accession no. PV012473), *Streptomyces ardesiacus* SSPJ32 (accession no. PV012474), and *Streptomyces tendae* SSPJ48 (accession no. PV012475). The sequences were deposited in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov).

Enzyme production in submerged fermentation. The crude enzyme was obtained through submerged fermentation (SmF) using 1% (w/v) paddy straw as the carbon source. The crude filtrate was subsequently assayed for enzyme activity against various substrates to evaluate its cellulolytic and hemicellulolytic potential, including exoglucanase (FPase), endo-glucanase, xylanase, and endo-arabinase activities (Fig. 1). FPase activity ranged from 0.037 to 0.052 U/mL, indicating the isolates' ability to hydrolyse crystalline cellulose. Endoglucanase activity, which reflects the degradation of amorphous cellulose, ranged from 0.16–0.26 U/mL. Xylanase activity, a key indicator of hemicellulose degradation, ranged from 0.71–0.81 U/mL, whereas endo-arabinase activity, which targets arabinan side chains in hemicellulose, ranged from 0.27–0.56 U/mL. Statistical analysis revealed significant differences in enzyme activity among the isolates, with *Streptomyces griseoincarnatus* SSPJ4 demonstrating higher enzymatic efficiency than the other strains. Furthermore, *S. griseoincarnatus* SSPJ4 was selected for whole-genome sequencing to identify genes encoding cellulase and hemicellulase.

Whole-genome analysis. Whole-genome sequencing of the *Streptomyces griseoincarnatus* SSPJ4 was performed using the Illumina platform, and de novo assembly, along with all subsequent analyses, was conducted using the Galaxy platform (https://usegalaxy.org). The assembled genome size was 7.3 Mbp with a ~72% GC content. The assembly process generated 533 contigs with an N50 value of 244,046. The genome assembly and annotation data were deposited in the NCBI GenBank under accession no. PRJNA1230074. Functional annotation and gene

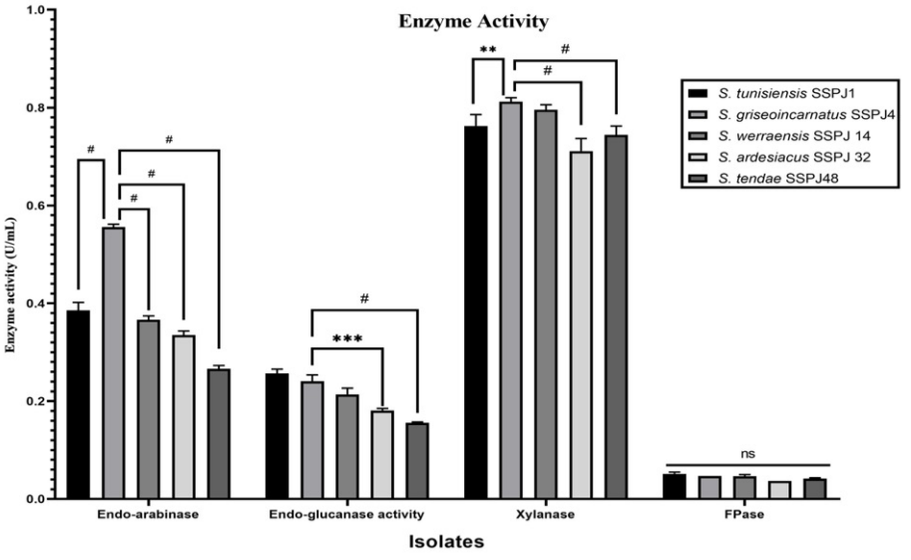

Fig. 1. Hydrolytic enzyme profile of *S. griseoincarnatus* SSPJ4 on paddy straw (1%) as the sole carbon source (# p<0.0001, ***p<0.005, **p<0.01, ns-non significant).

prediction were performed using the PROKKA pipeline on the Galaxy platform, which identified 6,678 genes. Among these, genes encoding cellulases, xylanases, and other hydrolytic enzymes were present, which align with the observed enzyme activities, as they are crucial for lignocellulosic biomass degradation. The general characteristics of the genome assembly and annotation are summarized in Table 1.

CAZy annotation. CAZyme analysis of the genome sequence of *S. griseoincarnatus* SSPJ4 was performed to evaluate its capacity to produce enzymes involved in the degradation of complex carbohydrates. A total of 226 genes were annotated with CAZyme annotation (Fig. 2). Of these, 91 were hypothetical proteins and 53 were cellulolytic and accessory enzymes, of which more than 50% had signal peptides indicating extracellular enzyme production (Table 2). This highlights the potential of this strain for efficient lignocellulosic biomass degradation.

Phylogenomic analysis of *Streptomyces griseoincarnatus* SSPJ4. In silico multilocus sequence typing (MLST) analysis was performed to determine the allelic profile of the isolate. The analysis revealed a novel combination of alleles across housekeeping genes: *16S rRNA*, *atpD*, *gyrB*, *recA*, *rpoB*, and *trpB*. The allelic profile of *S. griseoincarnatus* SSPJ4 was represented as *16S* (~146), *atpD* (~91), *gyrB* (~46), *recA* (~45), *rpoB* (~170), and *trpB* (52). This unique allelic profile suggests that the isolate represents a distinct sequence type (ST) within the *Streptomyces* genus, underscoring its genetic novelty and taxonomic significance. According to TYGS typing, the isolate

Table 1. Characteristic of *S. griseoincarnatus* SSPJ4 genome

Features	***S. griseoincarnatus* SSPJ4**
Total sequence length	73,64,574
Total length (≥ 0 bp)	74,76,771
Total length ((≥ 1000 bp)	73,58,507
Contigs	74
Contigs (≥ 0 bp)	533
Contigs (≥ 1000 bp)	64
Largest contig	5,48,685
GC (%)	72.38
N50	2,44,046
N90	56,132
auN	2,55,770.9
L50	11
L90	32
N's per 100kb	40.18
Contigs	498
Bases	74,65,395
CDS	6,589
gene	6,678
rRNA	4
tRNA	84
tmRNA	1

belonged to the known species *S. griseoincarnatus* (Fig. 3), as previously identified by 16S rRNA sequencing using EzBioCloud data. Digital DNA-DNA hybridization (dDDH) was performed to confirm the taxonomic identity of the strain. The dDDH values d4 for *Streptomyces griseoincarnatus* SSPJ4 and *S. griseoincarnatus* JCM 4381 were 95.5%. A comparative genomic technique was used to generate circular chromosomes. A map of the linear chromosome of *S. griseoincarnatus* SSPJ4 was created using the CG View server (Fig. 4).

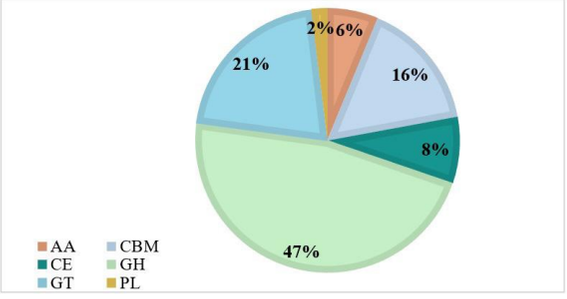

Fig. 2. CAZy count of *S. griseoincarnatus* SSPJ4

Biosynthetic gene clusters. The genome of *S. griseoincarnatus* SSPJ4 contains 23 biosynthetic gene clusters (BGCs) encoding pathways for the production of various secondary metabolites. These included clusters for terpenes, antibiotics, siderophores, geosmins, and butyrolactones (Fig. 5). Notably, many of these BGCs exhibit less than 20% similarity to known compounds in existing databases, suggesting the potential for novel secondary metabolites to be discovered. This low similarity highlights the genetic novelty of *S. griseoincarnatus* SSPJ4 and its potential to produce unique bioactive compounds with applications in medicine, agriculture, and industry.

To determine the ability of the isolates to produce enzymes on different substrates, enzyme production on different biomasses was performed for 15 days. *S. griseoincarnatus* SSPJ4 produced a fair amount of enzymes on all substrates, *namely*, wheat bran (WB), paddy straw (PS), pearl millet (PM), and sugarcane bagasse (SCB) (Fig. 6). Maximum enzyme activity was observed on day 5. FPase activity was 0.0557, 0.0687, 0.0499, and 0.0462 U/mL for WB, PS, PM, and SCB, respectively (Fig. 6A). The endo-glucanase

Table 2. Cellulolytic genes identified in *S. griseoincarnatus* SSPJ4 by CAZy annotation

S. N.	Product	EC#	Family
1	Lytic cellulose monooxygenase	1.14.99.54	AA10+CBM2, AA10
2	Multifunctional esterase	3.1.1.-	CE1
3	Bifunctional xylanase/deacetylase		CE4 (44-158)
4	Beta-glucosidase A	3.2.1.21	GH1
5	Bifunctional beta-D-glucosidase/beta-D-fucosidase	3.2.1.21	GH1
6	Endo-1,4-beta-xylanase A	3.2.1.8	GH10, GH11
7	Glycosyl hydrolase family 109 protein 1	3.2.1.-	GH109
8	Endo-1,4-beta-xylanase B	3.2.1.21	GH11
9	Alpha-amylase	3.2.1.1	GH13_32
10	Beta-galactosidase/hexosaminidase	3.2.1.23	GH2, GH3, GH42
11	Beta-hexosaminidase	3.2.1.52	GH20, GH3
12	Beta-xylosidase	3.2.1.-	GH3, GH43_36+CBM91
13	Alpha-xylosidase	3.2.1.177	GH31_3
14	Alpha-galactosidase AgaA	3.2.1.22	GH36
15	Beta-xylosidase	3.2.1.37	GH39, GH3
16	Putative 6-phospho-beta-glucosidase	3.2.1.86	GH4 (2-177)
17	L-arabinofuranosidase	3.2.1.55	GH5_35, GH62, GH51_1, GH51_2, GH43_12
18	Exo-beta-1,3-glucanase	3.2.1.58	GH55_1 (290-574)
19	Endoglucanase	3.2.1.4	GH6, GH5_2, GH12, GH4, GH9
20	Glucan endo-1,3-beta-glucosidase	3.2.1.39	GH64, GH16
21	Xylan alpha-(1->2)-glucuronosidase	3.2.1.131	GH67
22	Xyloglucanase	3.2.1.-	GH74
23	4-alpha-glucanotransferase	2.4.1.25	GH77

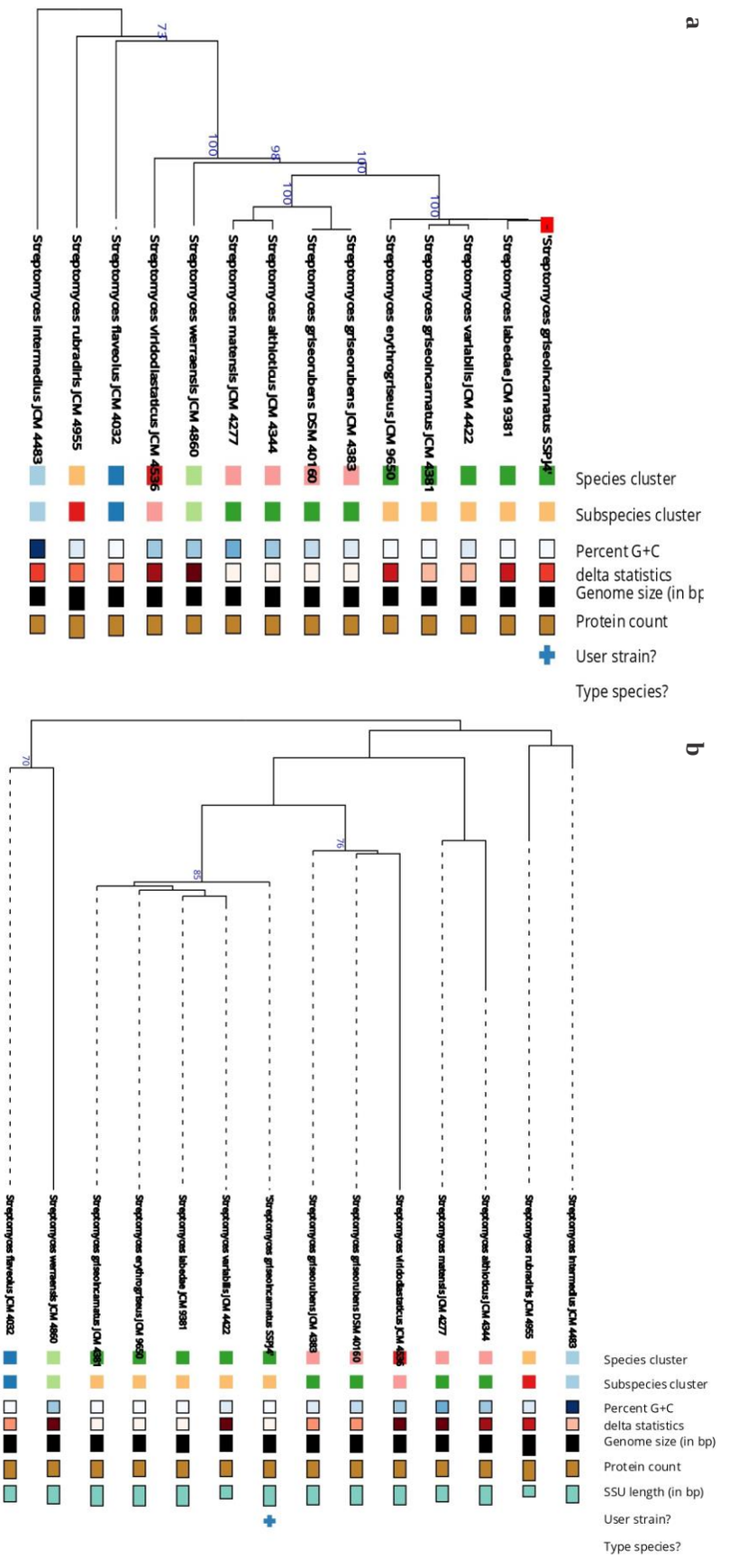

Fig. 3. TYGS GBDP Phylogeny of *S. griseoincarnatus* SSPJ4 based on (a) 16s data, (b) whole-genome data

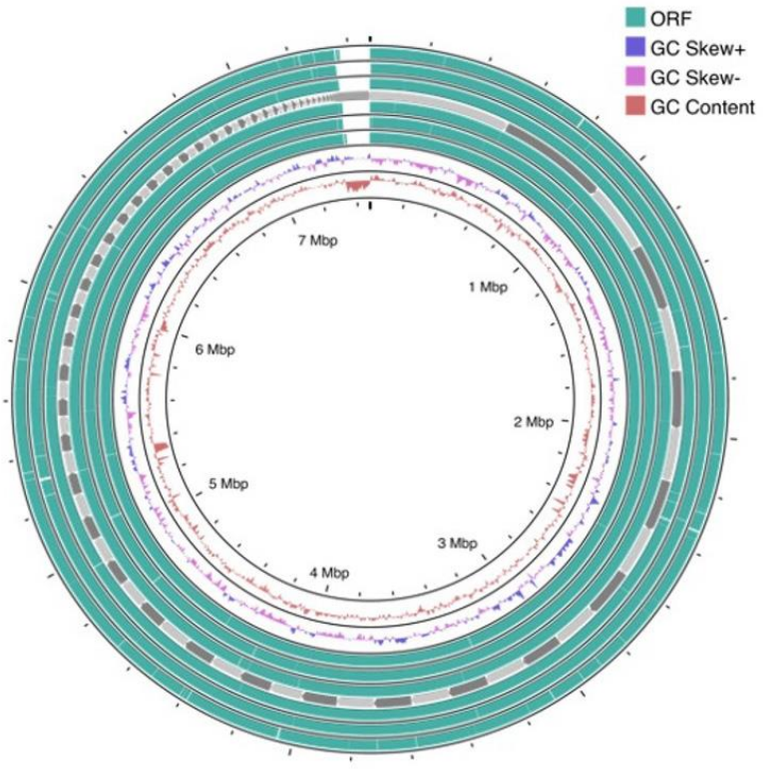

Fig. 4. Schematic representation of the linear chromosome of *S. griseoincarnatus* SSPJ4 created using the CG View server. Circles 1-3 display the ORFs. Circle 4 shows the CDS. Circle 8 shows a GC percentage plot. Circle 9 shows the GC skew.

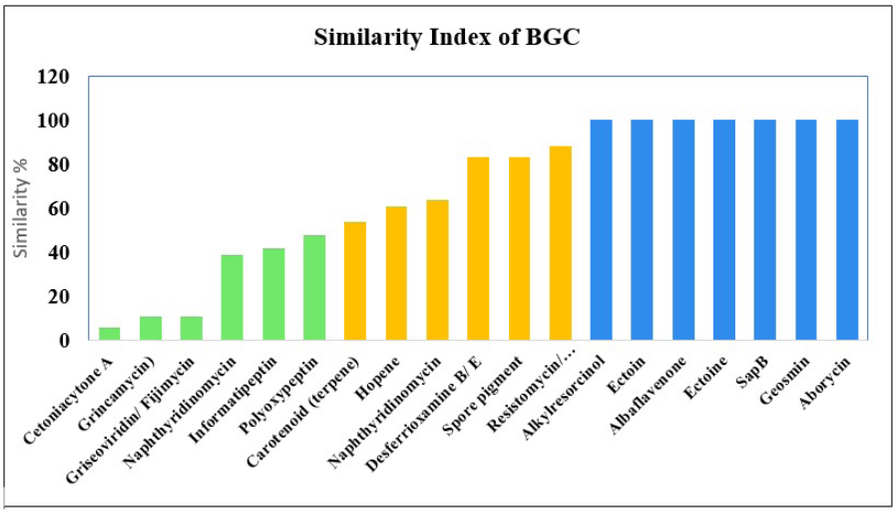

Fig. 5. Putative biosynthetic gene clusters from the genomes of *S. griseoincarnatus* SSPJ4 by antiSMASH

activities were 0.066, 0.194, 0.061, and 0.145 U/mL for WB, PS, PM, and SCB, respectively (Fig. 6B). The xylanase activities were 0.669, 0.685, 0.643, and 0.658 U/mL for WB, PS, PM, and SCB, respectively (Fig. 6C). The arabinase activities were 0.165, 0.196, 0.203, and 0.180 U/mL for WB, PS, PM, and SCB, respectively (Fig. 6D). Among the four substrates screened, paddy straw produced the maximum cellulase when fermented with *S. griseoincarnatus* SSPJ4 using SmF. However, a considerable amount of enzyme was produced in wheat bran.

The pretreated paddy straw was subjected to saccharification using the concentrated culture filtrate of *S. griseoincarnatus* SSPJ4 for 72 h at 50°C. The enzyme

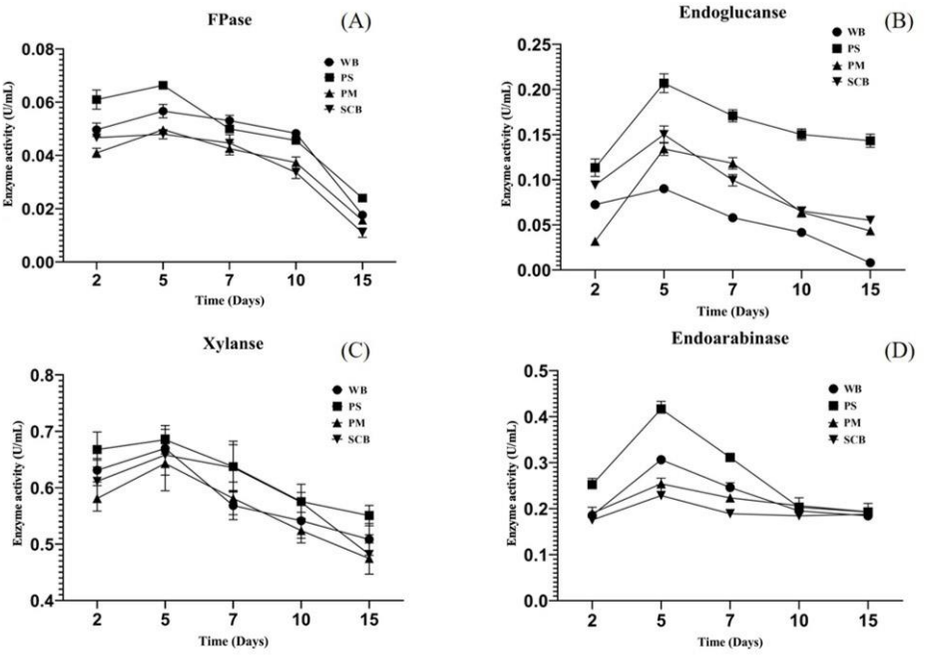

Fig. 6. Enzymatic profile of *S. griseoincarnatus* SSPJ4 for 15 days on wheat bran, paddy straw, pearl millet, and sugarcane bagasse: (A) FPase, (B) endo-glucanase, (C) xylanase, and (D) arabinase. [Wheat Bran (WB), Paddy Straw (PS), Pearl Millet (PM), Sugarcane bagasse (SCB)

Saccharification of pretreated paddy straw using an enzymatic cocktail.

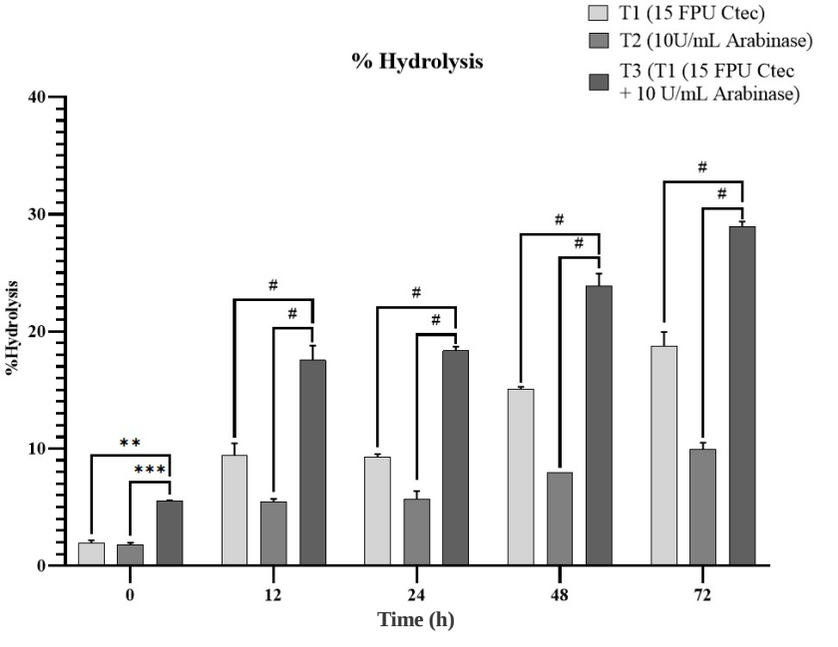

Fig. 7. Saccharification yield of pretreated paddy straw using various treatments of commercial cellulase and Indigenous enzyme ($p<0.0001$, ***$p<0.005$, **$p<0.01$)

(Treatments: T_1 – 15 FPU Celluclast®; T_2 – 10U/mL arabinase from *S. griseoincarnatus* SSPJ4; T_3 - Cocktail of 15 FPU Celluclast® + 10U/mL arabinase from *S. griseoincarnatus* SSPJ4)

activity of the concentrated secretome of *S. griseoincarnatus* SSPJ4 was FPase (1.4 U/mL), endoglucanase (2.68 U/mL), xylanase (12.67 U/mL), and endo-arabinase (4.34 U/mL). The experiment comprised three different treatment sets (Fig. 7). However, the enzyme activity of Celluclast® was FPase (93.18 U/mL), endo-glucanase (21.07 U/mL), xylanase (76.57 U/mL), and endo-arabinase (35.34 U/mL). When *S. griseoincarnatus* SSPJ4 was used alone, the saccharification yield was 9% after 72 h, which was relatively low compared to the other treatments. The highest saccharification yield of 29% was achieved when indigenous crude filtrate from *S. griseoincarnatus* SSPJ4 was used with the commercial cellulase Celluclast®. In contrast, 18.8% sacccharification yield, was obtained using 15 FPU of Celluclast®. Notably, the inclusion of indigenous crude enzymes containing arabinase significantly enhanced the saccharification yield, resulting in a 1.55-fold increase compared to Celluclast® alone.

DISCUSSION

In this study, we isolated and explored the genomes of cellulolytic actinomycetes from soil and degraded wood samples from the semi-arid region of Mahendergarh district, Haryana, India. Actinomycetes are frequently isolated from semi-arid areas owing to their ecological adaptation, making them suitable candidates for prospecting industrially important metabolites such as enzymes and antibiotics (23, 24). Actinomycetes have adapted to utilize recalcitrant substances, such as lignocellulosic biomass and xenobiotics, owing to their ability to produce diverse hydrolytic enzymes, including cellulase (25). Fungi have been used traditionally for the commercial production of many industrial enzymes, including cellulases, due to well- recognized advantages like high enzyme titers and diversity of enzymes. Most commercially available cellulases are of fungal origin (26). However, fungi are deficient in accessory enzymes such as arabinases, making actinomycetes suitable candidates for arabinase prospecting for application in different industries, including bioethanol production (27). Five isolates exhibiting cellulase and hemicellulase enzymes were identified as *Streptomyces* species based on 16S rRNA sequencing. Among the five identified *Streptomyces* species, *S. griseoincarnatus* SSPJ4 exhibited significantly higher activities of endoglucanase (0.26 U/mL), xylanase (0.81 U/mL), and endo-arabinase (0.56 U/mL), making it the most promising candidate for the production of these enzymes. The xylanase and cellulase production by *Streptomyces* sp. ranges from 0.4 to 0.8 U/mL and from 0.006 to 0.033 U/mL, respectively (28, 29), which is consistent with the results of the present study. Similarly, cellulase and xylanase production by *S. thermocoprophilus* TC13W yielded maximum specific activities of 925 and 1796 U/g, respectively (30). Waheeb et al. (2024) reported a specific activity (4.21 U/mg) of the purified enzyme from *S. thermodiastaticus*, which is consistent with the present study. The activity observed in the present study was consistent with that of previous studies, indicating the presence of robust lignocellulose-degrading genes (31). Considering the promising enzyme yields, especially of endo-arabinase, further genome mining of *S. griseoincarnatus* SSPJ4 was conducted to understand its genetic machinery and discover novel hydrolytic enzymes that can enhance its application in biotechnological processes, such as biofuel production and waste management. The genome size of *S. griseoincarnatus* SSPJ4 was 7.3 MB, with ~72% GC content, which is consistent with other *Streptomyces* species, which typically have a size range from 7-12 MB, and GC content ranges from 60-80% (32-35). The high GC content may contribute to their stability and the production of secondary metabolites. *S. griseoincarnatus* R-35, a marine isolate with a genome size of 7.6 Mb and GC content of 72.2%, has been previously reported (14). The N50 value was 2,44,046, which indicated that half of the sequence was present in one contig. However, this shows a moderate-quality assembly and is still considered reliable, especially when using short-read sequencing technologies such as Illumina. These genomic features provide a valuable resource for exploring the biotechnological potential of this strain, particularly in biofuel production and industrial enzyme development.

CAZyme automated annotation identified a diverse array of CAZymes, including 16 auxiliary activities (AA), 41 carbohydrate-binding modules (CBM), 121 glycoside hydrolases (GH), 54 glycosyl transferases (GTs), and five polysaccharide lyases (PLs). Similarly, *Streptomyces* sp. MS2A contains 184 genes belonging to the GH family, which hydrolyse glycosidic bonds of glycosides, resulting in free sugar production (36). The CAZy annotation predicted various hydrolytic enzymes from the GH family,

such as cellulase, xylanase, glucosidase, amylase, and arabinofuranosidase. The combination of cellulase, hemicellulase, and accessory enzymes in the genome of *S. griseoincarnatus* SSPJ4 highlights the potential of this isolate for producing hydrolytic enzymes. The CAZy annotation revealed that four arabinase enzymes were present in the genome *S. griseoincarnatus* SSPJ4, which belong to the GH family viz., GH43, GH51 (2), and GH5. The endo-arabinase isolated from *Bacillus licheniformis* belongs to the GH43 family (37).

Enzymatic hydrolysis is a one of the cost-intensive steps in bioethanol production. The use of accessory enzymes, high substrate loading, simultaneous saccharification, and fermentation are some of the strategies suggested for improving the sugar yield during saccharification. The saccharification of alkali-pretreated paddy straw using concentrated crude enzymes from *S. griseoincarnatus* SSPJ4 provided essential insights into its enzymatic activity. The 1.55-fold increase in saccharification yield compared to that commercial cellulase alone highlights the potential of supplementing enzyme cocktails with accessory enzymes like arabinase contained in the crude enzyme. Supplementation with accessory enzymes has been reported to enhance saccharification yields by 1.3 to 2-fold (38, 39). This indicated that the culture filtrate of *S. griseoincarnatus* SSPJ4 acted synergistically with commercial Celluclast® and improved the sugar release. Enzyme synergy in lignocellulosic biomass degradation plays a crucial role in enhancing the overall yield of fermentable sugars (40, 41). Arabinase breaks the hemicellulose side chain, hindering cellulase accessibility (42). These findings align with those of previous studies demonstrating enhanced saccharification efficiency when cellulase is combined with a hemicellulase. Using indigenous enzymes with accessory enzyme activities, such as arabinase from *S. griseoincarnatus* SSPJ4, can help achieve higher saccharification yields, making the process economically viable. Furthermore, optimizing enzyme combinations based on substrate composition can further enhance the overall efficiency of lignocellulosic biomass conversion.

CONCLUSION

The global energy demand continues to increase with the growing population and depletion of non-renewable energy resources, which can be replaced by second-generation biofuels. In the present study, a potential cellulolytic isolate, *S. griseoincarnatus* SSPJ4, was isolated, which efficiently hydrolysed cellulose and hemicellulose from lignocellulosic biomass. The adaptability of *S. griseoincarnatus* SSPJ4 to the semi-arid region indicates the presence of robust enzymes, making it a potential candidate for industrial application. The genomic insight further reveals the presence of various metabolites. CAZy annotation revealed around 50 cellulose and hemicellulose degradation enzymes, including accessory enzymes, which enhance the saccharification. The synergistic action of the indigenous accessory enzyme containing endo-arabinase from *S. griseoincarnatus* SSPJ4 with commercial cellulase increased the sugar titer compared to cellulase alone. Despite these promising results, further optimization, scaling-up, and strain improvement are required. However, with the availability of genomic data, genome engineering can be employed further to enhance the efficiency and titer of the various enzymes. Future studies will focus on optimizing enzyme production and exploring its potential for industrial applications.

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