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Optimization of 1, 4-α-D-glucan glucanohydrolase production from a novel strain *Aspergillus niger* MUL-14: isolation and purification of expression cassettes encoding

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Abstract

A novel soil isolate, MUL-14, was recognized as a hyper-producer of 1, 4-α-D-glucan glucanohydrolase (GGNH, EC 3.2.1.1), encoded by *amy* genes. GGNH is primarily used for starch liquefaction and has diverse applications in many industries. The study aimed to produce cost-effective GGNH from MUL-14 via solid-state fermentation. The internal transcribed spacer (ITS) sequence of MUL-14 was compared with other known sequences and the strain was identified as *Aspergillus niger*. The yield of gDNA was achieved 51.2-540.1 µg. Different solid substrates i.e., oat straw (*Avena sativa*), sugarcane bagasse (*Saccharum officinarum*), wheat straw (*Triticum aestivum*), rice straw (*Oryza sativa*), mustard oil cake (*Brassica napus*), and gram testa (*Cicer arietinum*) along with different moistening agents i.e., 0.05 M $C_2H_3NaO_2$, 0.05 M Na_3PO_4, 1N HCl, 150 mM NaCl (saline solution), distilled water, and tap water were evaluated individually without starch supplementation. The optimum production of the enzyme 765±1.327 U.gds^{-1} (LSD~0.192) and total protein contents 19.3±0.17 mg.gds^{-1} (LSD~5.389) were achieved when 10 g *Avena sativa* at 100 % moisture level (maintained with saline solution) was inoculated with 0.6×10^7 spores/ml (inoculum size of 5 %) for 72 h, at 30 ^oC, and pH 6.0. A high level of expressed cassettes of genes *amyA* (3325 bp) and *amyB* (3685 bp) were isolated and purified for further industrial strain development by genetic manipulation. This economically produced enzyme has great biotechnological potential in the food and textile industries.

Keywords: 1, 4-α-D-glucan glucanohydrolase · solid state fermentation · agro-industrial waste residues · *Aspergillus niger* · gDNA isolation · expression cassettes.

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1. INTRODUCTION

Alpha-1, 4-D-glucan glucanohydrolases (GGNHs, synonym: α-amylase, EC 3.2.1.1) randomly breaks α-1, 4 glycosidic linkages in starch polymer chains to liberate maltose, malto-triose, and short-chain α-limit dextrin. GGNH can neither break the α-1, 6 linkage branch point, nor the terminal glucose residue as it is a non-debranching endo-amylase. In the present-day biotechnology, this enzyme has immense importance as it has been used in diverse areas of health such as analytical, clinical, pharmaceutical, and drug industries. In addition to these, the enzyme is used: in the industrial production of glucose, maltose, and

high fructose corn syrups, in bakery industries for producing aroma, taste, and better smoothness of the bread by affecting anti-salting, in detergent industries for improving bleaching efficiency, in brewage industry for the production of fermentable sugar through the conversion of cereal and tuber starches which are then fermented to ethyl alcohol, in textile industries for the desizing of fabrics, and in paper industry to improve the quality and hardness of paper and to protect against mechanical injury [1, 2].

In addition to plants and animals, microorganisms are the most potent sources of GGNHs. However, compared to bacterial GGNHs, fungal enzymes are more stable and yield higher enzyme titre when growing on natural biological substrates [2]. Yuan et al. [3] identified 17 novel plant polysaccharides hydrolysing enzymes belonging to glycoside hydrolase families i.e., GH13, GH15, and GH31, which were previously unknown in the genome of *A. niger*. Among those a gene (*amyC*) product has shown a significant starch hydrolysing activity which was non-inducive and independent of transcriptional regulator (*amyR*: a gene encoding amylolytic transcription regulator). GGNH belongs to family GH13 [4]. Genomic DNA studies of *A. niger* var. *awamori* indicated two copies of a gene encoding for GGNH, designated as *amyA* and *amyB*. Both iso-genes are arranged as eight introns and nine exons. The nucleotide sequences of both genes are identical except for the last few nucleotides of their respective coding regions [5].

A. niger is the most commonly used fungus for commercial amylase production because it has high acid tolerance ability hence, bacterial contamination can easily be avoided in the culture medium. The United States Food and Drug Administration has approved it as a GRAS strain therefore, these enzymes encoded by the genome of this fungus can be used in the food industry. Furthermore, the hyphal mode of growth facilitates the deep penetration into the solid particles, increasing the availability of nutrients to the growing fungus hence, SSF is the best mode of fermentation to achieve a high yield of enzymes from fungal sources [1, 6–8]. SSF is also adopted for the biological pre-treatment of biomass by filamentous fungi such as corn cobs [9], corn stover [10], corn straw [11], cotton stalks [12], and oat straw [13]. The fungal strain, particle size, fermentation temperature, moisture contents, and fermentation time were optimized to improve the quality of oat straw biomass [13].

In SSF, the solid substrate acts as a source of nutrients e.g., rice bran comprises 34 % carbohydrates and fat, protein, crude fibre, and minerals i.e., potassium, calcium, and phosphorus in reasonable amounts [14]. Similarly, rice husk is comprised of carbohydrates such as hemicellulose (25 %), lignin (20 %), and cellulose (35 %) [15]. In addition to nutrient availability, culture conditions strongly influence the development of mold and enzyme secretion [16]. In SSF, mold development, and product yield are strongly affected by the initial moisture level of the solid substrate. The porosity among the substrate particles decreases with increasing moisture content hence, the oxygen exchange rate also decreases. A low moisture content leads to a decrease in substrate utilization resulting in sub-optimal organism growth, reducing product formation. The most favourable moisture level depends upon the type of substrate and the organism involved [17, 18]. Similarly, microbes are very sensitive to pH and temperature changes for their optimal growth. Initial spore concentrations also affect the nutrient utilization rate in solid media during fermentation; hence, the enzyme yield is also affected [19, 20].

Only a few chosen strains of bacteria and fungi match the requirements for commercial production, even though GGNH has been used in starch-based businesses for many decades and may be produced efficiently from various microbiological sources. The pursuit of novel microbes capable of producing GGNH is an ongoing endeavour. Hence, the present study aimed to evaluate different solid substrates, moistening agents, and culture conditions to achieve optimal production of GGNH from a novel strain. It was further aimed to isolate and purify expression cassettes (genes with their native promoter and terminator sequences) from a novel hyper-producer *A. niger* MUL-14.

2. MATERIALS AND METHODS

2.1 Chemicals

All the chemicals used in this study were of analytical grade and purchased directly from E-Merck (Germany), Sigma (USA), Acros (Belgium), BDH (UK), and Fluka (Switzerland).

2.2 Isolation, screening, identification of strains, and inoculum preparation

The novel strain *Aspergillus niger* MUL-14 was isolated by serial dilution method, from humus soil samples collected from the parking area of Minhaj University Lahore. All the procedures for screening and morphological identification of the soil isolates, and inoculum preparation as spore suspension of *A. niger* MUL-14 were carried out as described previously [21].

2.3 Extraction of genomic DNA (gDNA)

One milliliter of inoculum was added to 99 ml of FGFM containing ($g.L^{-1}$) starch 20, lactose 10, yeast extract 8.5, corn steep liquor 6.0, $MgSO_4.7H_2O$ 1.2, NH_4Cl 1.3, and $CaCl_2.2H_2O$ 0.6 (pH 5.0). The flasks were incubated at 30 ^oC for 8 h with constant agitation (200 rpm). Young mycelia were harvested using sterilized filter papers (Whatman® grade 1) and washed with chilled sterilized distilled water. gDNA was extracted in accordance with previous method [22], with some modifications. Mycelia were ground to a fine powder using liquid nitrogen. CTAB (pH 8.0) buffer (2.5 ml) containing (%, w/v) CTAB 2, Tris-HCl 1.55, EDTA 0.7, NaCl 8.15, and β-mercaptoethanol 1 (%, v/v) was mixed with 300 mg of powdered mycelia. A mixture (625 µl) was added into sterilized Eppendorf tubes separately. The tubes were incubated at 65 ^oC for 30 min and cooled on ice for 3-5 min. An equal volume (625 µl) of phenol:isoamyl alcohol:chloroform (25:24:1) was added into individual tubes. The tubes were vortexed vigorously and centrifuged at 13,000 rpm (25,900×*g*) for 10 min. The upper layer was transferred into other Eppendorf tubes, separately. Iso-propanol and absolute ethanol (167 µl of each) were then added to individual tubes. The tubes were vortexed and incubated at -20 ^oC for 1 h, afterward, the same samples were centrifuged for 10 min at 13,000 rpm (25,900×*g*). The supernatants were discarded and the pellets were re-suspended in 200 µl of 70 % ethanol. The tubes were vortexed and again centrifuged as described above. The pellets were dried at room temperature by inverting the tubes on a paper towel. Air-dried pellets were dissolved in 25 µl of 1X (pH 8.5) TE buffer (10 mM Tris-Cl and 1 mM EDTA). One microliter of RNase A (10 $mg.ml^{-1}$) was added, mixed, incubated at 37 ^oC for 15 min, centrifuged as described above, and stored at -20 ^oC. The extracted DNA was resolved on an agarose gel (0.8 %) parallel to a DNA ladder (Gene Ruler™, Fermentas, catalog number SM0313) containing ethidium bromide. The yield of gDNA was measured by taking absorbance at 260 nm using Thermo Scientific NanoDrop1000. The purity of the isolated gDNA was confirmed by calculating the absorbency ratio A_{260}/A_{280}.

2.4 Amplification and sequencing of the rRNA gene complex

The ribosomal RNA (rRNA) gene complex, incorporating 18S small subunit complete sequence, ITS1, 5.8S rRNA gene, ITS2, and 28S large subunit partial sequence encoded by rDNA was amplified by using gDNA as template and universal primers (ITS-1-F 5′-TCCGTAGGTTGAACCTGCGG-3′, ITS-4-R 5′-TCCTCCGCTTATTGATATGC-3′) [23]. A PCR reaction mixture (25 µl) was prepared containing 2 µl gDNA (5 $ng.\mu l^{-1}$), 2.5 µl $MgCl_2$ (25 mM), 2.5 µl *Taq* buffer (10X), 2.5 µl dNTPs (2.5 mM), 1 µl each of the primers (10 $pmol.\mu l^{-1}$, Invitrogen), 0.25 µl *Taq* DNA polymerase (5 $U.\mu l^{-1}$), and 13.25 µl deionized water. rDNA was amplified by conducting 25 cycles, each of which consisted of three steps namely, denaturation (95 ^oC, 1 min), annealing (55 ^oC, 1 min), and extension (72 ^oC, 1 min) using a thermal cycler (Bio-Rad C1000). Initial denaturation and final elongation were performed at 95 and 72 ^oC, respectively for 5 min. Amplicons were fractionated on a gel (0.8 % agarose). The fragments were purified from the agarose gel using Favor Prep PCR Clean-Up Mini Kit (FAPCK 001-1) according to the manufacturer's instructions and submitted for sequencing to Eurofins MWG Operon USA. The obtained sequence was submitted to the GenBank database (accession number OQ581729.1). NCBI $Blast_n$ was performed to determine the nucleotide sequence homology (http://www.ncbi.nlm.nih.gov/BLAST). rDNA sequences representing the rRNA gene complex including the ITS regions of *A. niger* var. *awamori* were aligned by MUCSLE using the UPGMA method. Molecular evolutionary analyses were carried out by constructing a phylogenetic tree (MEGA X).

2.5 Amplification of *AmyA* and *AmyB* expression cassettes

The primers used for the amplification of expression cassettes Amyl 1 of *amyA* (accession number AM270278.1, locus_tag: An12g06930) and Amyl 2 of *amyB* (accession number AM270106.1, locus_tag: An05g02100) [24] were designed (Table 1) as described previously [25]. The gDNA of *A. niger* MUL-14 was used as a template. Reaction mixture which had a total volume of 25 µl was comprised of 2.5 µl 10X *Taq* buffer in 16.25 µl MilliQ containing 2.5 µl of $MgCl_2$, 1 µl of gDNA, 0.5 µl of dNTPs, 0.5 µl of *Taq* polymerase and 1 µl each of the primers viz., Amy1 flanking primer forward (Amy1_F_F), Amy1 flanking primer reverse

(Amy1_F_R) and Amy2 flanking primer forward (Amy2_F_F), Amy2 flanking primer reverse (Amy2_F_R) for the amplification of complete expression cassettes of *amyA* (3a) and *amyB* (3b) genes (Table 2). To minimize sequencing errors both expression cassettes were amplified in short fragments (1a, 2a, 1b, and 2b) using internal primers viz., Amy internal primer forward (Amy_I_F), and Amy internal primer reverse (Amy_I_R) whose sequence is conserved in both genes (Fig. 1; Table 2) by overlap extension PCR (Fig. 2). For the 1st round of the reaction mixtures (25 µl) were designed as: 2.5 µl 10X *Taq* buffer in 16.25 µl MiliQ containing $MgCl_2$ 2.5 µl, gDNA 1.2 µl, dNTPs 0.5 µl, *Taq* polymerase 0.25 µl and 1 µl each of primers. In 1st round, the reactions were carried out for amplicons 1a, 1b, 2a and 2b as shown in Table 3. For 2nd round, the contents from tube 1 and tube 2 were transferred into tube 3, and overlapping was carried out at 65 °C for 1 min (Fig. 2). For the 3rd round, the reactions were carried out using flanking primers under the same conditions for amplicons 3a and 3b (whole genes) as depicted in Table 3. PCR products were resolved on agarose gel (0.8 %) parallel to the DNA ladder. Favor Prep PCR Clean-Up Mini Kit (FAPCK 001-1) was used to purify PCR products which were subsequently submitted for sequencing.

Table 1. Primers used for the amplification of expression cassettes encoding for 1, 4-α-D-glucan gluconohydrolase from *A. niger* MUL-14.

Primer ID	Primer sequence	Melting temperature (T_m)
Amy1_F_F	GGCTTCTAGGCGCGCTCCATC	70 °C
Amy1_F_R	TTATGATATAGCTCCTCTCCAAGC	68 °C
Amy_I_F	TGCAACTGACTTCGCGGATATGG	70 °C
Amy_I_R	CGTCATGGACGGCGTACTGAAC	70 °C
Amy2_F_F	CGCACTACCCGAATCGATAGAAC	70 °C
Amy2_F_R	CGTATACCTGTCTTGTGTTAGGC	68 °C

Table 2. Size of amplicons encoding for 1, 4-α-D-glucan gluconohydrolase from *A. niger* MUL-14.

Amplicon ID	Forward primer ID	Reverse primer ID	Amplicon size (bp)
1a	Amy1_F_F	Amy_I_R	2202
2a	Amy_I_F	Amy1_F_R	2389
3a	Amy1_F_F	Amy1_F_R	3354
1b	Amy2_F_F	Amy_I_R	1945
2b	Amy_I_F	Amy2_F_R	2239
3b	Amy2_F_F	Amy2_F_R	3687

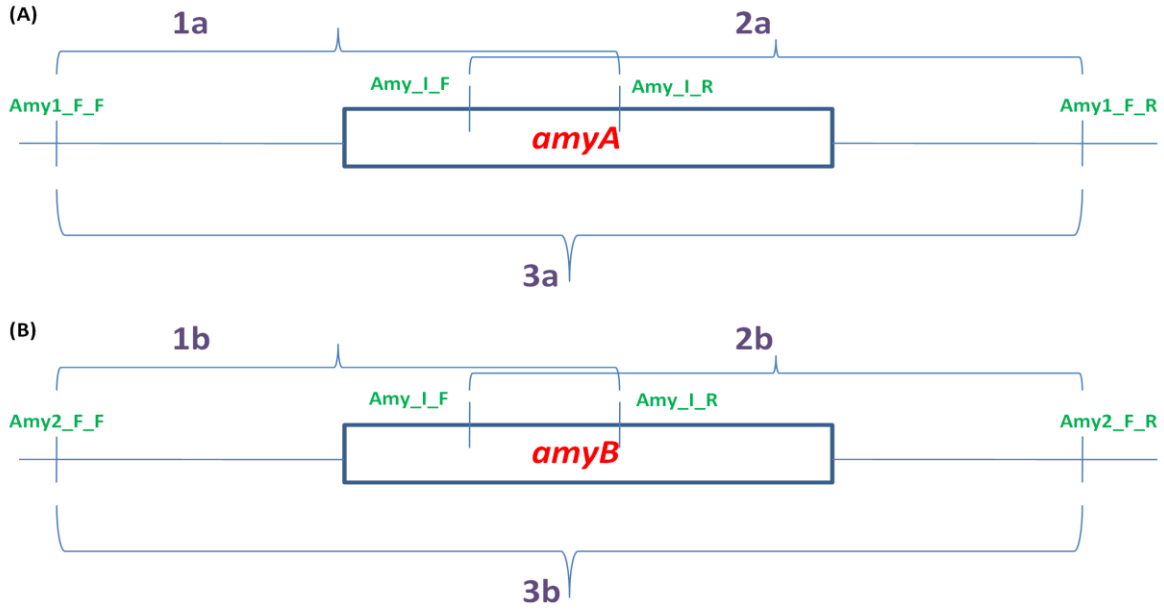

Fig. 1. Schematic representation for the amplification of expression cassettes (A) *amyA*, (B) *amyB* from *A. niger* MUL-14. Square boxes (bold line) are showing gene sequences. Narrow lines at the anterior and posterior ends of both boxes are showing promoter and terminator sequences of the expression cassettes. Primer's IDs are written in green color. 1 and 2 are showing half sequence while 3 is showing a full sequence of both expression cassettes *amyA* and *amyB* each designated as a and b.

Table 3. Polymerase chain reaction (PCR) conditions for the amplification of expression cassettes encoding for 1, 4-α-D-glucan gluconohydrolase from *A. niger* MUL-14.

Amplicon ID	Initial denaturation		Denaturation		Annealing		Extension		Final extension	
	Temp. (^oC)	Time (min)	Temp. (^oC)	Time (min)	Temp. (^oC)	Time (min)	Temp. (^oC)	Time (min)	Temp. (^oC)	Time (min)
1a	95	3.30	95	0.30	65	1.0	72	2.0	72	10
2a	95	3.30	95	0.30	64	1.0	72	2.0	72	10
3a	95	3.30	95	3.30	64	1.0	72	3.15	72	10
1b	95	3.30	95	0.30	65	1.0	72	2.0	72	10
2b	95	3.30	95	0.30	64	1.0	72	2.0	72	10
3b	95	3.30	95	3.30	64	1.0	72	3.30	72	10
-	-		← 35 cycles →						-	

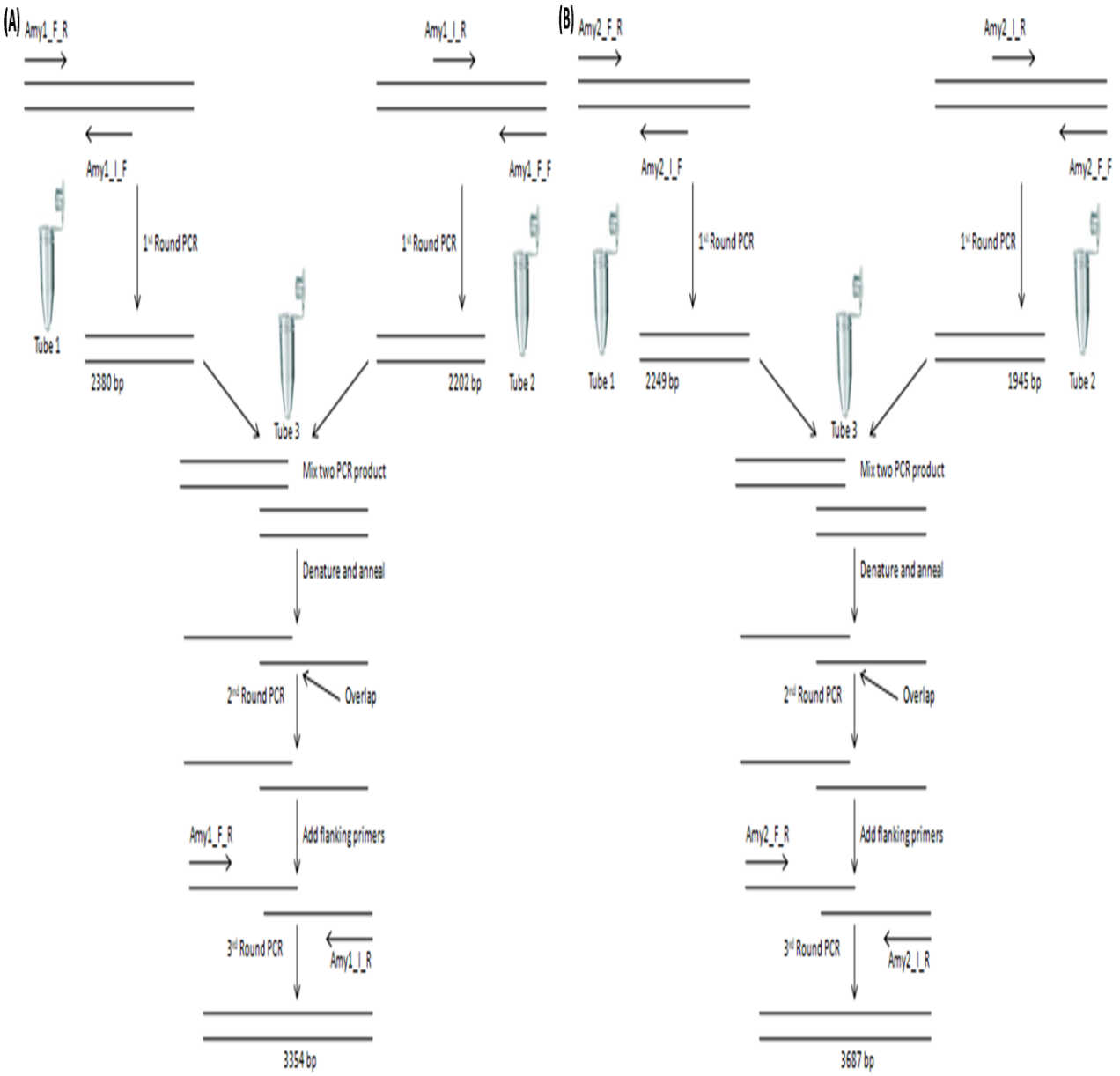

Fig. 2. Amplification of expression cassettes. In 1st round one internal and one external primers were used to amplify the half sequence of both iso-genes in individual PCR tubes. In 2nd round, the contents of tube 1 and tube 2 containing the PCR product were mixed in tube 3 and the PCR was carried out without primers to overlap the half-sequence fragments of each iso-gene. In 3rd round, only external primers were used for the amplification of the full length of each gene expression cassette.

2.6. Solid State Fermentation (SSF)

Agricultural waste biomass residues (sugarcane bagasse (*Saccharum officinarum*), mustard oil cake (*Brassica napus*), oat (*Avena sativa*), gram testa (*Cicer arietinum*) rice straw (*Oryza sativa*), and wheat straw (*Triticum aestivum*)) was used as a low-cost solid substrate (Fig. 3) for the cultivation of *A. niger* MUL-14. All the substrates were incubated for 15 min individually at 80±2 ^{o}C and the dry weight was recorded. Ten grams of each solid substrate was soaked to 100 % moisture level by using 0.05 M $C_2H_3NaO_2$, 0.05 M Na_3PO_4, tap water, 1N HCl, 150 mM NaCl (saline solution), and distilled water, individually at pH 5.0. The initial moisture contents were calculated in accordance with Kunamneni et al. [26] as follows:

$$Moisture\ contents\ (\%) = \frac{wt.of\ solid\ substrate - dry\ wt.}{dry\ wt.} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

The flasks were sterilized at 15 lbs/in² pressures, 121 ^{o}C for 15 min. Then they were cooled to room temperature (~25 ^{o}C) and inoculated (under aseptic conditions) with 1 ml of spore suspension separately

(prepared as mentioned above). The contents of each flask were mixed vigorously. All flasks were incubated for 72 h at 30 ^{o}C. Afterward, the enzyme was extracted in 50 ml of (0.05 M) Na_3PO_4 buffer (pH 7.0) by shaking (150 rpm) for 120 min, at 30 ^{o}C. The flask contents were squeezed through muslin cloth and centrifuged (13,000 rpm; 25,900×*g*) at 4 ^{o}C for 15 min. The resulting clear supernatant was used as a crude enzyme source [27]. For optimization, static cultures were incubated at various temperatures (20-55 ^{o}C), pHs (4.0-9.0), times of incubation (12-96 h), and inoculum sizes (0.5-3.0 ml). Spore counts were performed as described previously [28].

Fig. 3. Agricultural waste residues used as a solid substrate in SSF. (A) Wheat straw (*Triticum aestivum*), (B) Rice straw (*Oryza sativa*), (C) Sugar cane bagasse (*Saccharum officinarum*), (D) Oat straw (*Avena sativa*), (E) Gram testa (*Cicer arietinum*), (F) Mustard oil cake (*Brassica napus*).

2.7. Biochemical analysis

The enzyme assay was carried out according to Bernfeld [29] using soluble starch solution (1 %, w/v) as substrate. The blank and control reactions were also run parallel by substituting the enzyme and substrate with 1 ml of Na_3PO_4 buffer, respectively. All the tubes were incubated in a water bath at 60 ^{o}C, pH 7.0, and 100 rpm for 15 min. The amount of reducing sugar thus released was estimated by the DNS reagent [30]. The O.D. values were taken at 540 nm. The data was computed in a linear regression equation to calculate maltose concentration as a standard. One unit (U) of the active enzyme was defined as "the amount of GGNH that liberated 1 µmol maltose under specified assay conditions". The enzyme units were then converted to $U.gds^{-1}$. The total protein content was estimated by Bradford reagent [31] and converted to $mg.gds^{-1}$.

2.8. Statistical Analysis

The data were analysed by employing the protected LSD method, one-way ANOVA, and Duncan's multiple range test using SPSS-v20, and presented in the form of *p*-value ($\alpha = 0.05$) [32].

3. RESULTS AND DISCUSSIONS

3.1. Molecular identification of MUL-14

The sequencing of the rRNA gene complex originated a fragment length of approximately 599 bp. This result is in accordance with *Aspergillus niger* isolate S-1 (GenBank accession number OQ520279.2). The sequence was aligned by BLASTn with 99-100 % coverage, and accommodated lengths 599 - 1699 bp. The percentages of sequences alignment with MUL-14 are shown in Table 4. The lengths of the aligned sequences of *Aspergillus niger* isolates varied between 598-76751 bp. The sequences of the rRNA gene complex of the nuclear-encoded rDNA showed significant percentage identities (98.83 %) with those of MUL-14. Twenty-nine rRNA gene complex sequences of the genus *Aspergillus* subgenus *Circumdati* section *Nigri* were aligned by MUSCLE and a phylogenetic tree was constructed (Fig. 4). The sum of the branch lengths was calculated as 44.05414693. The percentages of replicate trees in each cluster are shown next to the branches. The alignment results and phylogenetic analysis of the rRNA gene complex revealed that MUL-14 belongs to the genus *Aspergillus* subgenus *Circumdati* section *Nigri* and species *niger*.

Table 4. Ribosomal RNA gene sequence complex of novel fungal isolate have significant alignment with other ITS sequences of genus *Aspergillus* sub-genus *Circumdati* section *Nigri* *.

No.	Strains	No. of sequences aligned (n) out of 100 shown (%)	Percentage identity (%)	Acc. Length	E-value
1.	*Aspergillus niger*	65	98.83	598-76751	0.0
2.	*Aspergillus welwitschiae*	08	98.83	625-1699	0.0
3.	*Aspergillus awamori*	15	98.83	625-1182	0.0
4.	*Aspergillus* sp.	09	98.83	599-639	0.0
5.	*Aspergillus foetidus*	02	98.99-98.66	614-623	0.0
6.	*Aspergillus niger* MUL-14	1	100	599	0.0

*Nucleotide sequence alignment was performed by BLASTn.

3.2. Evaluation of solid substrates and moistening agents

Among all the agricultural waste residues studied as substrates for the best GGNH production, *Avena sativa* (oat) with 530±0.99 U.gds^{-1} (total protein contents 16.9 mg.gds^{-1} (LSD~0.267)) was evaluated as the best substrate for GGNH production and varied significantly (LSD~2.651) from the other substrates (Fig. 5A). The decreasing order of tested solid substrates based on enzyme activity was: oat>oil cake>gram testa>sugarcane bagasse>rice straw>wheat straw. Diverse carbohydrate sources distinctly exaggerate enzyme synthesis. Therefore, different agricultural waste residues have been evaluated as substrates for the production of GGNH from different fungal sources (Table 5). Hence, different solid substrates have been optimized with different fungal strains for GGNH production. For example, Balakrishnan et al. [45] and Padmini et al. [40] optimized groundnut oil cake, and sugarcane bagasse as the best substrates for GGNH production from *A. oryzae* and *A. flavus*, respectively whereas, Suganthi et al. [38] and Mathew et al. [44] optimized coconut (*Cocos nucifera*) oil cake as the superior source of nutrient solid medium for the production of the same enzyme.

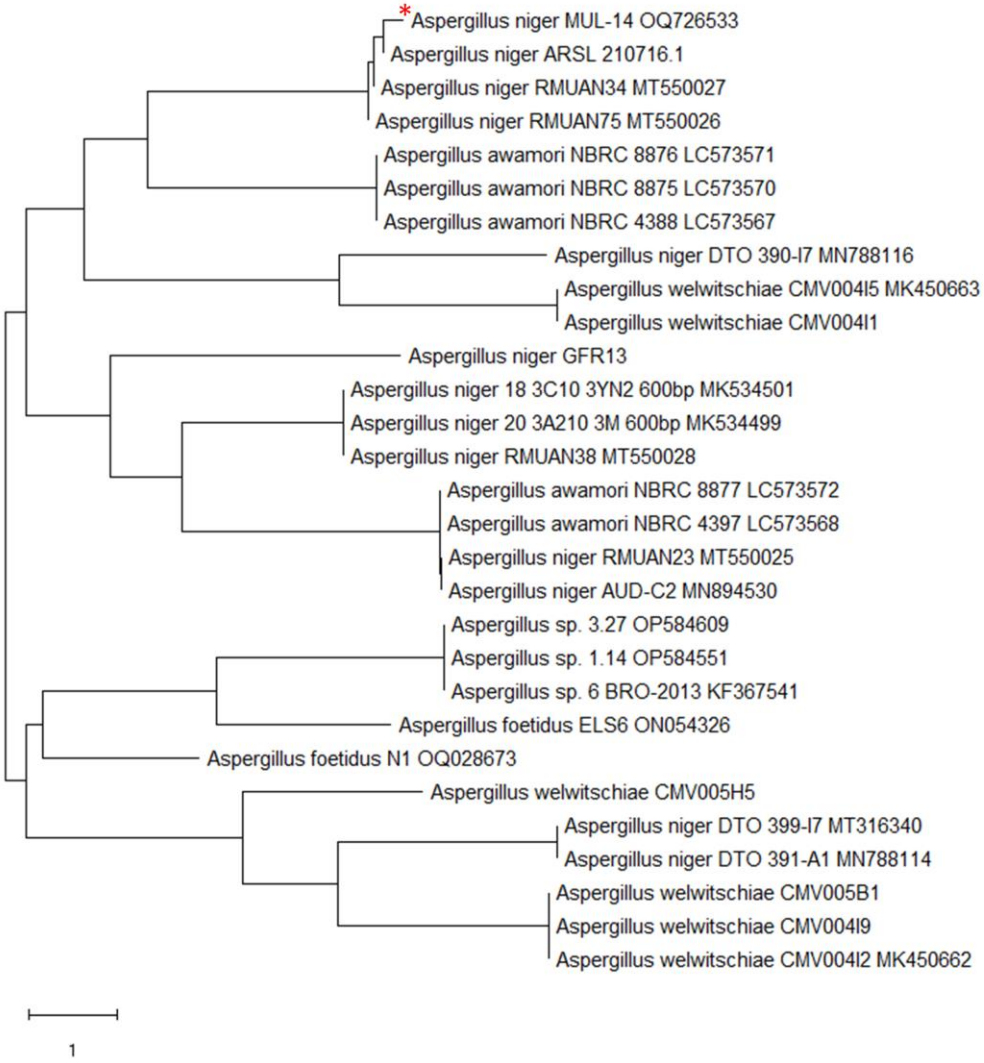

Fig. 4. The phylogenetic tree was inferred using the Neighbor-Joining method. The branch lengths were calculated by the Maximum Composite Likelihood method. Evolutionary analyses were carried out by MEGA-X. Novel isolate *Aspergillus niger* MUL-14 is indicated by red asterisk (*).

Among the moistening agents saline solution (0.00877g.ml^{-1} NaCl) was found to be the best for GGNH production (680±1.0 U.gds^{-1}) and significantly more effective (LSD~2.66) than the other moistening agents used (Fig. 5B). The decreasing order of moistening agents with respect to enzyme activity was as follows: saline solution>sodium phosphate buffer>1N HCl>distilled water>sodium acetate buffer>tap water. The hyphae of fungi owing to their ability to cyclize in the cytoplasm, have the capacity to develop and expend. They have excellent tolerance to high osmotic pressure and low moisture contents [46]. However, these characteristics are strongly affected by the type of moistening agent which ultimately affects GGNH production from different fungal sources.

Therefore, different moistening agents viz., 0.1 M acetate buffer, phosphate buffer, and distilled water have been evaluated for the production of GGNH [37]. Puri et al. [42], and Kaur et al. [47] optimized mineral salt solution as the best diluent for the production of GGNH from *A. oryzae* and *Rhizopus oryzae*, respectively. However, in the present study, *Avena sativa* (oat) and saline solution were optimized for subsequent experimentation.

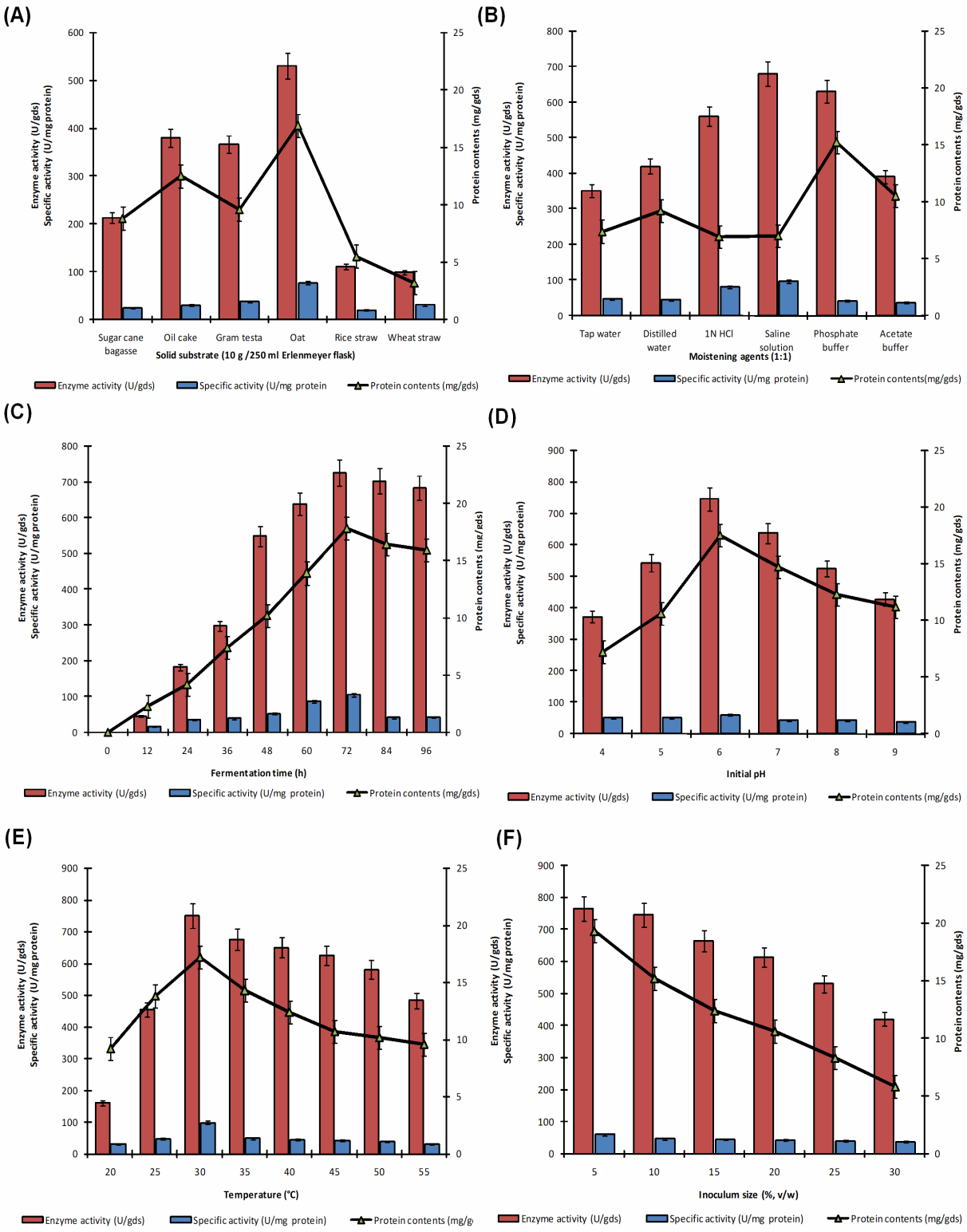

Fig. 5. Optimization of culture conditions for the production of 1,4-α-D-glucan glucanohydrolase from *A. niger* MUL-14 by SSF, (A) Effect of different agro-industrial waste residues, (B) Effect of different moistening agents, (C) Effect of incubation time, (D) Effect of initial pH, (E) Effect of temperature, (F) Effect of inoculums size.

3.3. Optimization of cultural conditions

To optimize the rate of enzyme production, fermentation was carried out for 96 h (Fig. 5C). The enzyme activity gradually increased with time, and the optimum yield was 725±3.465 $U.gds^{-1}$ (LSD~3.615) after 72 h. The total protein contents were 17.8±0.13 $mg.gds^{-1}$ (LSD~2.32). By further increasing the incubation time (84-96 h), a gradual decrease in enzyme activity was detected. Microbial growth involves different growth stages during fermentation.

Table 5. Agro-industrial waste residues used as solid substrates for the production of 1, 4-α-D-glucan gluconohydrolase by SSF.

No.	Organism	Solid substrates	Reference
1.	*Aspergillus niger* NRRL-337	Cane molasses, glucose syrup, rice bran, corn bran, corn Starch, wheat bran.	[33]
2.	*Humicola lanuginose*	Wheat bran, rye straw, wheat straw.	[34]
3.	*Aspergillus niger* MTCC-104	Wheat bran, soybean meal, rice bran, and black gram bran.	[35]
4.	*Aspergillus niger*	Vegetable waste (potato, tomato, brinjal), wheat bran, banana peel and rice husk.	[36]
5.	*Trichothecium roseum*	Sunflower oil meal, corncob leaf, wheat bran, rye straw and rice husk.	[37]
6.	*Aspergillus niger* BAN3E	Gingely oil cake Rice bran, coconut oil cake, black gram bran, wheat bran, and groundnut oil cake.	[38]
7.	*Aspergillus flavus*	Molasses bran, sugarcane bagasse, rice bran, wheat bran and maize meal.	[39]
8.	*Aspergillus flavus*	Corn cob, rice straw, rice bran, and sugarcane bagasse.	[40]
9.	*Aspergillus flavus*	Wheat bran, maize meal, rice bran, molasses bran and sugarcane bagasse.	[41]
10.	*Aspergillus oryzae*	Rice bran, wheat bran, wheat bran: rice bran and (1:1) and paddy husk: rice bran (1:1).	[42]
11.	*Aspergillus niger* sp.CMST04	Black gram bran, rice bran, corn waste, and wheat bran.	[43]
12.	*Aspergillus niger*	Rice bran, wheat bran, gingerly oil cake, groundnut oil cake, black gram bran, and coconut oil cake.	[44]
13.	*Aspergillus niger* MUL-14	Sugar cane bagasse, *Brassica napus* oil cake,oat straw, gram testa, rice straw, and wheat straw.	In this study

At each stage, different primary and secondary metabolites are produced at different rates. Therefore different incubation times have been reported which specifically depend upon the type of strain used. Ramachandran et al. [48] and Sivaramkrishnan et al. [49] optimized 72 h for optimal enzyme production from *A. oryzae* which is in accordance with our finding for the cost-effective production of the enzyme. However, Geetha et al. [50] and Irfan et al. [51] optimized 96 h for GGNH production from *A. flavus*, and *A. niger*-ML-17 respectively, and even longer periods i.e., 108, and 120 h were optimized for *A. oryzae,* and *A. flavus* enzymes, respectively [40, 45]. Hence, the production of enzymes from these strains increases the cost of production. Further, the longer incubation times may also cause problems such as contamination and the formation of large amounts of diverse by-products.

At pH 4.0, the GGNH activity was very low then it was increased to 543±4.665 $U.gds^{-1}$ at pH 5.0 and reached a maximum 745±2.05 $U.gds^{-1}$ (LSD~3.877) at pH 6.0 (Fig. 5D). The total protein contents were estimated to be 17.5±0.019 $mg.gds^{-1}$ (LSD~0.096). Similarly, Mamatha et al. [41] optimized the pH 6.0 when they were working with *A. flavus*. However, Varalakshmi et al. [52] reported a pH of 9.5 for the best GGNH production from *A. niger* GJI-24. Figure 5E shows that the optimum activity of GGNH was 750±2.15 $U.gds^{-1}$ at 30 ^{o}C (LSD~2.184) with total protein contents of 17.2±1.9 $mg.gds^{-1}$ (LSD~0.0931) then after which a gradual decrease in enzyme activity was observed from 35 to 55 ^{o}C. Similarly, Kaur et al. [47] reported that 30 ^{o}C was best for GGNH production from *R. oryzae* which is well aligned with our study. A relatively high optimal temperature value of 70 ^{o}C for the same enzyme from *A. niger* MTCC-104 was also reported [35]. *A. niger* MUL-14 preferred slightly acidic pH and ambient temperature for its growth which reduces the cost and energy requirements. Hence, this strain is well suited for the production of GGNH at the industrial scale.

The inoculum size was also varied (5-30 %) by inoculating *Avena sativa* (oat) medium with 0.5 to 3.0 ml of spore suspension individually (Fig. 5F). The spore concentrations used for each inoculum size are depicted in Table 6.

Table 6. Spore concentration in different inoculum sizes used for the production of 1, 4-α-D-glucan gluconohydrolase from *Aspergillus niger* MUL-14 by SSF.

No.	Spore suspension (ml)	Inoculum size (%)	Spores.ml^{-1}
1.	0.5	5	0.6×10^7
2.	1.0	10	1.2×10^7
3.	1.5	15	1.8×10^7
4.	2.0	20	2.4×10^7
5.	2.5	25	3.0×10^7
6.	3.0	30	3.6×10^7

The best enzyme activity (765±1.327 U.gds^{-1} (LSD~0.192)) and total protein contents (19.3±0.17 mg.gds^{-1} (LSD~5.389)) were achieved when 5 % inoculum was used. In accordance with our findings, a 5 % inoculum size for the production of GGNH from *R. oligosporus*-ML-10 and *A. niger*- ML-17 has also been reported [51].

However, Premalatha et al. [2] optimized 2.5 % inoculum of *Aspergillus tamarii* MTCC5152, and Balkan et al. [37] used 10 % inoculum of *Trichothecium roseum* for the best production. The use of a lower initial concentration of spores is best because a higher concentration not only utilizes a greater amount of nutrients but also produces a large amount of microbial biomass that lowers the yield of the product. The spores per ml used by other workers are given in Table 7 for comparison.

3.4. Purification and Sequencing of Expression Cassettes

The high level of expression of the *amyA* and *amyB* genes in the SSF of *A. niger* MUL-14 encouraged technologists to develop recombinant strains for industrial applications. Hence, these genes were isolated and purified along with their native promoter and terminator regions. The intact band of extracted gDNA, on 0.8 % agarose showed good quality gDNA (Fig. 6 A and B). The yield of gDNA from *A. niger* MUL-14 was 51.2-540.1 µg/300 mg wet mycelia. The A_{260}/A_{280} ratio was in the range of 1.87-1.91, indicating that the extracted gDNA was free of impurities. The expression cassette of amylase 1 (3325 bp) comprised of 670 bp *amyA* promoter, 2,096 bp *amyA* gene, and a 559 bp *amyA* terminator while amylase 2 (3687 bp) comprised of 883 bp *amyB* promoter, 2,026 bp *amyB* gene, and 778 bp *amyB* terminator regions, were amplified (Fig. 6 A and B).

Table 7. Comparison of spore concentrations used for the production of 1, 4-α-D-glucan gluconohydrolase by SSF.

No.	Organism	Spores/ml	References
1.	*Aspergillus flavus var.columnaris*	0.0637×10^6	[53]
2.	*Aspergillus oryzae*	8×10^7	[54]
3.	*Aspergillus niger* USM AI 1	1×10^4	[55]
4.	*Aspergillus niger* ATCC 16404	5×10^6	[56]
5.	*Penicillium janthinellum* NCIM 4960	5×10^7	[57]

6.	*Aspergillus niger and Aspergillus flavus*	5×10^5	58
7.	*Neurospora crassa* CFR 308	1×10^7	17
8.	*Aspergillus niger*	5×10^4	59
9.	*Aspergillus flavus*	1×10^7	41
10.	*Rhizopus oryzae*	1×10^7	47
11.	*Aspergillus tamarii* MTCC5152	1×10^6	2
12.	*Aspergillus niger* MUL-14	0.6×10^7	In this study

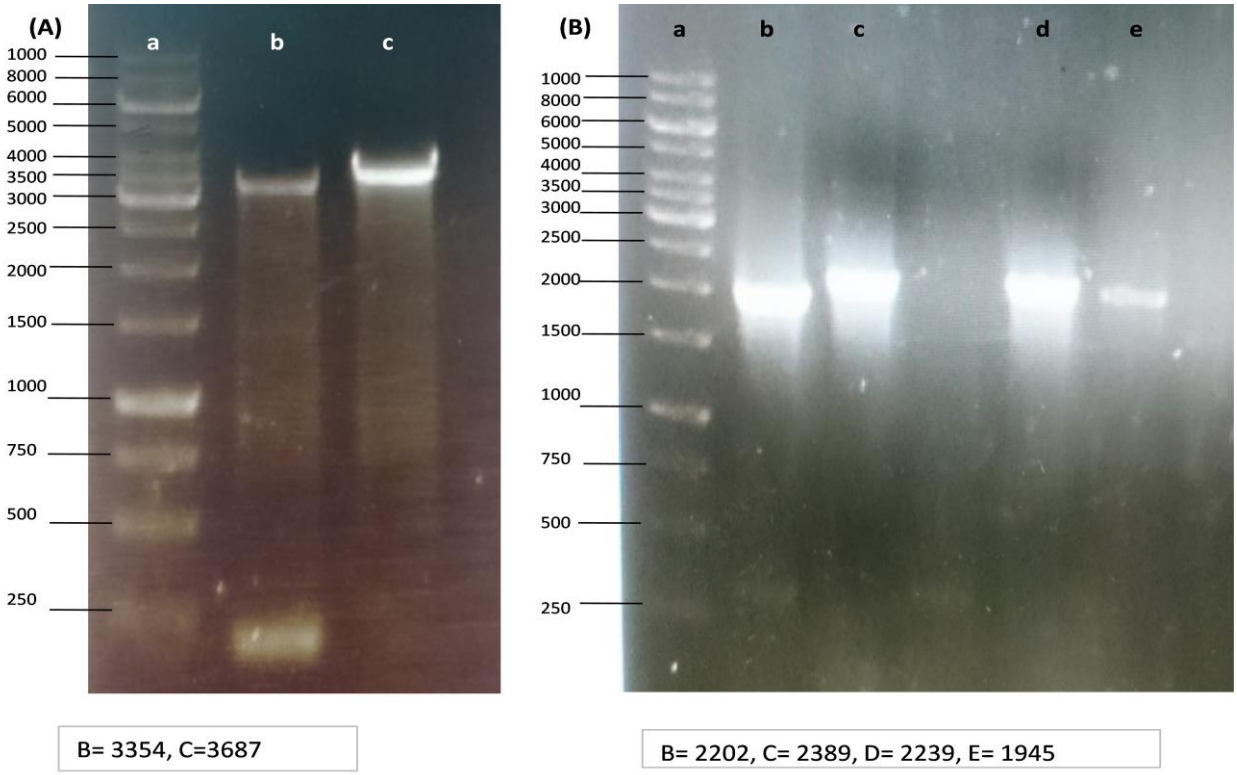

Fig. 6. Amplification of expression cassettes *amyA* and *amyB* (A) (a) Gene Ruler™, (b)-3a (3325 bp), (c)-3b (3687 bp), (B) (a) Gene Ruler™, (b) 1a (2202 bp), (c) 2a (2389 bp), (d) 1b (2239 bp), (e) 2b (1945 bp).

3.5. Future aspects

The isolated GGNH expression cassettes from *A. niger* MUL-14 will be manipulated to construct an efficient vector for the cloning and overexpressing of *amy* gene/s in *Saccharomyces cerevisiae* to make a versatile industrial strain capable of converting starch into fuel ethanol in a single step by releasing fermentable sugar through liquefaction.

4. CONCLUSIONS

It is concluded that agricultural waste residues are promising natural sources of nutrients for the growth of fungi in SSF. These residues can be used as solid substrates in simple and cost-effective ways for the sustainable production of high-value industrial products. The optimization of SSF's culture conditions significantly increased the enzyme production. *A. niger* MUL-14 isolate is a potent source of GGNH. Hence, the expression cassettes encoding GGNH were isolated for further genetic manipulation.

NOMENCLATURE

GGNH = 1, 4-α-D-glucan glucanohydrolase
MUL-14 = Minhaj University Lahore strain no. 14
ITS = Internal transcribed spacer sequence
gDNA = Genomic deoxyribonucleic acid
SSF = Solid-state Fermentation
AmyR = the transcriptional regulator of starch-degrading enzymes
GRAS = generally recognized as safe
FGFM = Fungal growth fermentation medium
CTAB = Cetyltrimethylammonium bromide
EDTA = Ethylenediaminetetraacetic acid
MUCSLE = Multiple sequence comparison by log-expectation
UPGMA = unweighted pair group method with arithmetic mean
MEGA X = Molecular Evolutionary Genetics Analysis version X
DNS = 3, 5-Dinitrosalicylic acid
OD = Optical density
LSD = Least significant difference
ANOVA = Analysis of variance

NOVELTY STATEMENT

Aspergillus niger MUL-14 is a novel hyper-producer of GGHN. This study offers valuable insights into the effective use of agricultural waste residues for the cost effective production of GGNH for industrial applications.

AUTHOR'S CONTRIBUTION

Bilqees Fatima: Conception of the study, Supervision, performed the bench work for isolation, identification of strain and molecular studies, data analysis, writing an original draft, reviewed, edited, and approved the final draft; **Amir Nazeer**: performed the bench work for optimization experiments; **Muhammad Mohsin Javed**: Reviewed, edited, and approved the final draft.

CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

ETHICAL APPROVAL

Not applicable.

SUPPLEMENTARY INFORMATION

Not applicable.

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