Improvement of a Fungal Strain by Repeated and Sequential Mutagenesis and Optimization of Solid-State Fermentation for the Hyper-Production of Raw-Starch-Digesting Enzyme

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A selected fungal strain, for production of the raw-starch-digesting enzyme by solid-state fermentation, was improved by two repeated sequential exposures to γ-irradiation of Co⁶⁰, ultraviolet, and four repeated treatments with N-methyl-N'-nitrosoguanidine. The mutant strain Aspergillus sp. XN15 was chosen after a rigorous screening process, with its production of the raw-starch-digesting enzyme being twice that of usual wild varieties cultured under pre-optimized conditions and in an unsupplemented medium. After 17 successive subculturings, the enzyme production of the mutant was stable. Optimal conditions for the production of the enzyme by solid-state fermentation, using wheat bran as the substrate, were accomplished for the mutant Aspergillus sp. XN15. With the optimal fermentation conditions, and a solid medium supplemented with nitrogen sources of 1% urea and 1% NH₄NO₃, 2.5 mM CoSO₄, 0.05% (v/w) Tween 80, and 1% glucose, the mutant Aspergillus sp. XN15 produced the raw-starch-digesting enzyme in quantities 19.4 times greater than a typical wild variety. Finally, XN15, through simultaneous saccharification and fermentation of a raw rice corn starch slurry, produced a high level of ethanol with Yp/s of 0.47 g/g.

Keywords: Raw-starch-digesting enzyme, Aspergillus sp., repeated mutagenesis, optimal culture condition, ethanol production

The raw-starch-digesting enzyme (RSDE), composed of glucoamylase and other amylases, is capable of hydrolyzing raw starch into glucose, and other oligosaccharides, at mild temperatures and without requiring the conventional two-step process of liquefaction and saccharification for ethanol fermentation [13, 33]. The liquefaction of substrates before ethanol fermentation requires high energy for heating the raw products, and the use of RSDEs could reduce the total energy cost for ethanol production by approximately 30%. In addition, RSDEs could provide advantages in the conversion process of biomass into sugars, when compared with single enzyme acid hydrolysis, since it provides a higher quality end product, is energy efficient, provides for a safer working environment, and utilizes longer lasting equipment [18]. RSDE was found in fungal strains such as Aspergillus sp., A. usami, and Rhizopus sp. [1, 2, 18].

The improvement of microbial strains using different mutagenic agents for increased production of industrial products has been considered in the commercial fermentation process [26]. For instance, Thermomyces lanuginosus was treated with ultraviolet radiation (UV) and N-methyl-N'-nitrosoguanidine (NTG) to improve α-amylase and glucoamylase production [29]. The production of CMCase by F. oxysporum was improved after treatment with UV followed by NTG [15]. A. niger was treated with γ-irradiation of Co⁶⁰ for the improvement of glucoamylase production [27]. After treatment with UV followed by NTG, one mutant, Acremonium cellulolyticus, produced activities of FPase and β-glucosidase in quantities much greater than those found in its wild state [11]. In this study, various mutagenic agents such as γ-ray of Co⁶⁰, UV, and NTG were combined to mutate fungal strains with the aim of achieving hyperproduction of RSDE.

Both solid and submerged liquid fermentation systems have been used for enzyme production. Solid-state fermentation (SSF) has advantages when compared with submerged fermentation in that it is simpler, requires less capital, has superior productivity, has a lower energy requirement, requires simpler fermentation media, does not require rigorous control of fermentation parameters, uses less water, produces

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less wastewater, allows for the easy control of bacterial contamination, and has a lower cost requirement for downstream processing [5, 14, 23, 25].

For the cost-effective production of ethanol by simultaneous saccharification and fermentation, low-cost starchy substrates that produce high levels of RSDE are required. To date, only a few microorganisms producing RSDE are known or reported to be used in ethanol production. The purposes of this study were to attempt to go some way to rectifying this oversight by identifying a highly active microbial strain producing RSDE for the use in ethanol production, and then to significantly improve its RSDE activity by utilizing a new mutation method combining a variety of different mutagenic agents. The production of the RSDE was subsequently improved by optimization of SSF conditions, and finally the RSDE was tested for the production of a high concentration of ethanol from the raw starchy substrates.

**Materials and Methods**

**Isolation of Fungal Strain Producing RSDE**

Screening and selecting of fungal strains were conducted using PDA (potato dextrose, 2% agar) plates containing 0.1% of raw rice starch–Remazol Brilliant Blue (RBB), according to the method of Omemu et al. [21] with some modifications. Fungal strains were isolated from a variety of soils and fruits, and a number of other sources. One g of each sample was suspended in saline solution (0.85% NaCl) and diluted to $1 \times 10^{-7}$ g. The dilution (0.1 ml) was loaded onto a PDA plate containing raw rice starch-RBB, ampicillin (50 µg/ml), and tetracycline (50 µg/ml) and incubated at 30 °C for 3–7 days. Fungal strains that formed large clearing zones surrounding the colonies were picked up and then purified by streaking on a PDA plate, at 30 °C for 7 days. From the first samples, 14 RSDE-producing fungi (KV1~KV14) were selected for further study.

**Strain Improvement by Mutations**

Out of the 14 isolates, KV13 was found to be the most potent fungal strain for RSDE production, and this strain was used for subsequent strain improvement utilizing two methods of mutation.

**Method I.** The spores of fungal strain KV13 ($10^8$ spores/ml) were harvested from 6-day-old spores grown on PDA plates and exposed to different doses (0.5 to 2.5 Kgy with 0.5-Kgy interval) of γ-ray. The survivors were grown by spreading 0.1 ml of treated spores on a PDA plate containing 0.1% of raw starch–RBB and then incubated at 30°C for 4–7 days. Based on the size of clearing zones surrounding the colonies, γ-ray mutant X2 was chosen for further treatment with γ-ray of 2 Kgy. Following methodology from Kuhad et al. [15] with some modifications, the best performing γ-ray mutant X2-2 was continuously exposed to UV irradiation at 30 W in a distance of 50 cm for 5–60 min, where spores were taken out to test at 5-min intervals. The best mutant NU-1 selected was again simultaneously treated with NTG and UV (Fig. 2).

**Method II.** Selected fungal strain X2-2 obtained from Method I (Fig. 1) was used in Method II. The strain X2-2 was simultaneously treated with NTG (100 µg/ml) and UV at 30 W in a distance of 50 cm for 5–60 min, where spores were taken out to test at 5-min intervals. The best mutant NU-1 selected was again simultaneously treated with NTG and UV (Fig. 2).

**Fig. 1.** Repeated and sequential mutagenesis for improvement of RSDE production (Method I).

**Fig. 2.** Repeated and sequential mutagenesis with simultaneous treatment of NTG and UV for improvement of RSDE production (Method II).

The X2-2 mutant was obtained from Method I.
The selection of hyper-RSDE-producing mutants was based on the diameter of the clearing zone surrounding the colony on the plate-screening medium containing raw starch-RBB.

**Determination of RSDE Activity**

A 0.05 M sodium acetate buffer (pH 5.0) was used to dilute the enzyme aliquot and to suspend raw rice starch. The blank contained 0.5 ml of enzyme aliquot, 1 ml of 3.5-dinitrosalicylic acid (DNS), and 0.5 ml of 2% raw rice starch solution. The mixture reaction contained 0.5 ml of 2% raw rice starch solution and 0.5 ml of enzyme aliquot at 30°C for 30 min, at which time the reaction was stopped by adding 1 ml of DNS. Both the blank and the reaction mixture were then boiled at 100°C for 5 min, cooled, and 5 ml of distilled water was added to each, and the absorbance was measured at 540 nm. Reducing sugar as glucose was measured with Miller’s method [19], and 0.1% glucose solution (Sigma) was used to form a standard curve. One unit (U) of RSDE activity was defined as the amount of enzyme in 1 ml that liberates 1 µmole of glucose per min from raw rice starch [19].

**Identification of Fungal Strain**

The DNA of the fungal strain was isolated and purified using a Wizard Genomic DNA Purification Kit (Promega, U.S.A.). The identification of 28S rDNA was performed with the method of Henry et al. [12] and White [32]. PCR primers of ITS1 (5’-TCTTAAAGTTGAAACCTCTGCG-3’) and ITS3 (5’-GATCATGATGAAGAAGCCAGC-3’), and ITS4 (5’-TCTCCGGTCATTGATATGC-3’) [32] were used. PCR amplification was performed with a volume of 50 µl. Five µl of sample containing about 5 ng of DNA was added to the PCR master mixture, which consisted of 5 µl of 10× PCR buffer, 4 µl of dNTP mixture (0.1 mM each dNTP), 0.8 µl of each primer (40 pmol of each primer), and 2 units of ExTaq DNA polymerase (Takara Biomedicals, Osaka, Japan), and de-ionized distilled water to a final volume of 50 µl. Amplification consisted of denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 4 min; the Gene Amp, AB Applied Biosystem (Singapore) was used. PCR products were purified and sequenced. The sequence of the internal transcribed spacers (ITS1–ITS4) of 28S rDNA was aligned with well-known similar sequences from NCBI for identification.

**Preparation of Seeding Culture**

A 250-ml Erlenmeyer flask containing 10 g of wheat bran powder and 90 ml of distilled water, pH 3.5, was sterilized at 121°C for 20 min, and then cooled. To this, one plug (1×1 cm²) of 7-day-old conidia–mycelia was inoculated and incubated at 30°C in a shaker operated at 200 rpm for 1 day.

SSF

A 500-ml Erlenmeyer flask containing 20 g of wheat bran with 33.3% of moisture was plugged with cotton and sterilized for 20 min at 121°C. After cooling, 10% of 1-day-old liquid culture was inoculated and cultured at 30°C for 3 days.

**Preparation of Crude Enzyme**

The moldy wheat bran, after 3 days of fermentation, was mixed with distilled water at the ratio of 1:100 (w/v). The mixture was shaken in an orbital shaker at 200 rpm, at 30°C for 60 min. The supernatant obtained after centrifugation at 7,000 rpm for 10 min was used as crude enzyme.

**Optimization of SSF**

Various physicochemical parameters required for the optimal production of RSDE by the selected mutant XN15 were examined. Substrate (wheat bran, rice husk, rice bran, saw dust), moisture [20%, 30%, 40%, 50%, 60%, 70%, and 80% (v/w)], culture temperature (20, 25, 30, 35, 40, and 45°C), initial pH of solid culture (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0), culture time (2–6 days), aeration area (15, 20, 25, 30, 35, 40, and 45 g of moistened wheat bran in a 500-ml Erlenmeyer flask), age of seeding culture (1–4 days), and inoculum size [10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, and 50% (v/w)] were the parameters to be optimized. Studies were also conducted to examine the effect of various additives supplemented into the wheat bran solid culture on enzyme production. The examined additives were carbon sources of glucose, maltose, rice starch, sucrose, and corn, at 1% each; nitrogen sources of urea, yeast extract, tryptone, tryptic soy, peptone, nutrient broth, skim milk, casaminoacids, soytone, malt extract, NH4Cl, and NH4NO3, at 1% each; metal salts of CaCl2, MgCl2, MnCl2, CuSO4, CoSO4, FeSO4, ZnSO4, NaNO3, and KCl, at 2.5 mM each; surfactant of Tween 20, Tween 80, and Triton-X100, 0.5% each; and SDS and EDTA, at 0.4 mM each. SSF was carried out at 30°C for 3 days with wheat bran containing 33% of initial moisture, which was inoculated with 10% (v/w) of 1-day-old seeding culture. All experiments were conducted in triplicate. Commercial crude enzymes of Aspergillus usamii or Rhizopus sp. (Korea Enzyme Co., Korea) were used for comparison.

**Ethanol Production by Simultaneous Saccharification and Fermentation Using RSDE and *Saccharomyces cerevisiae***

Uncooked rice–corn [1:2 (w/w)] slurry, containing 35.78% (w/v) solid, and uncooked sweet potato slurry, containing 25.73% (w/v) solid, were used for simultaneous saccharification and fermentation. The simultaneous saccharification and fermentation was conducted in a 20-l jar containing 15 l of slurry, pH 3.5, with crude enzyme of isolated fungal strains with 1.13 Unit of RSDE/gds (gram dried substrate). To the slurry, yeast cells of *S. cerevisiae* KV25 were inoculated to make an initial cell number of 2.5×10⁹/ml, incubated at 30°C for 4–5 days, and the ethanol formed was determined. All experiments were conducted in triplicate.

**Determination of Ethanol**

The contents of ethanol were analyzed by HPLC. The column was Shodex SH1011 (8.0 mm ID×300 mm) and the mobile phase was 0.01 N sulfuric acid. Temperature was kept at 50°C with a flow rate of 0.6 ml/min and sample volume of 10 µl. The effluent from the column was monitored with an RI detector (Shodex RI-101).

**Statistical Program**

Data were analyzed using one and two way analyses of variance (ANOVA) (α=0.05) followed by comparison of means using the Duncan’s multiple range test (SAS Institute, Cary, NC, U.S.A.).

**RESULTS AND DISCUSSION**

**Screening and Selection of a Native Fungal Strain**

Many isolates were able to grow on the screening medium. Judging from the ratio between the clearing zone diameter...
and colony, 14 best isolates (KV1 to KV14) were chosen for enzyme production studies. The profiles of the enzyme production on the 3rd day by the selected 14 fungal strains are shown in Table 1. The activities of RSDEs produced by the fungal strains differed significantly \((P<0.005)\). The crude enzymes of fungal strains produced in solid wheat bran were used for ethanol production by simultaneous saccharification and fermentation using uncooked slurries of rice–corn starch or sweet potato (Table 2). Fungal strain KV13 showed a higher activity of RSDE \((30.11 \text{ U/ml})\) than those of the other fungal strains \((P<0.05)\) and its crude enzyme also produced the highest level of ethanol from uncooked rice–corn starch as well as uncooked sweet potato (Table 2). Therefore, strain KV13 was used for strain improvement by mutation.

### Table 1. The activities of RSDEs produced by various fungal strains.

<table>
<thead>
<tr>
<th>Fungal strain</th>
<th>RSDE (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KV1</td>
<td>9.82ecd</td>
</tr>
<tr>
<td>KV2</td>
<td>0.94e</td>
</tr>
<tr>
<td>KV3</td>
<td>19.30bc</td>
</tr>
<tr>
<td>KV4</td>
<td>7.53ecd</td>
</tr>
<tr>
<td>KV5</td>
<td>5.65eced</td>
</tr>
<tr>
<td>KV6</td>
<td>16.65bcd</td>
</tr>
<tr>
<td>KV7</td>
<td>25.13ba</td>
</tr>
<tr>
<td>KV8</td>
<td>5.63eced</td>
</tr>
<tr>
<td>KV9</td>
<td>30.06ba</td>
</tr>
<tr>
<td>KV10</td>
<td>5.31ecd</td>
</tr>
<tr>
<td>KV11</td>
<td>28.66ba</td>
</tr>
<tr>
<td>KV12</td>
<td>3.88ed</td>
</tr>
<tr>
<td>KV13</td>
<td>30.11a</td>
</tr>
<tr>
<td>KV14</td>
<td>18.04bcd</td>
</tr>
</tbody>
</table>

Statistical analysis \((P) <0.005\)

The means in the same column followed by same letters are not significantly different in Duncan’s multiple range test.

### Table 2. Ethanol production from the slurries of raw rice–corn starch and raw sweet potato by simultaneous saccharification and fermentation using crude enzymes of isolated fungal strains.

<table>
<thead>
<tr>
<th>Crude enzyme of fungal strain</th>
<th>Ethanol (% v/v) produced from slurry of*</th>
</tr>
</thead>
<tbody>
<tr>
<td>KV1</td>
<td>1.47e 6.08d</td>
</tr>
<tr>
<td>KV12</td>
<td>1.38e 1.60e</td>
</tr>
<tr>
<td>KV3</td>
<td>4.44d 8.28cb</td>
</tr>
<tr>
<td>KV4</td>
<td>2.34e 5.63d</td>
</tr>
<tr>
<td>KV5</td>
<td>0.64e 1.60e</td>
</tr>
<tr>
<td>KV6</td>
<td>2.15e 6.59cd</td>
</tr>
<tr>
<td>KV7</td>
<td>5.63de 8.96b</td>
</tr>
<tr>
<td>KV8</td>
<td>0.64e 1.74e</td>
</tr>
<tr>
<td>KV9</td>
<td>9.42e 8.37cb</td>
</tr>
<tr>
<td>KV10</td>
<td>0.64e 2.02e</td>
</tr>
<tr>
<td>KV11</td>
<td>6.59e 8.96b</td>
</tr>
<tr>
<td>KV12</td>
<td>0.74e 2.47e</td>
</tr>
<tr>
<td>KV13</td>
<td>13.72a 13.15a</td>
</tr>
<tr>
<td>KV14</td>
<td>0.64e 2.61e</td>
</tr>
</tbody>
</table>

Statistical analysis \((P) <0.0001\)

*Ethanol production by simultaneous saccharification and fermentation was carried out at 30°C for 5 days.

The means in the same column followed by same letters are not significantly different in Duncan’s multiple range test.

### Screening and Selection of a Mutant Strain

The production levels of RSDE by various mutant strains after treatment with different mutagenic methods are shown in Table 3. The improvement of enzyme production of mutant strains was dependant on the mutagenic method used, as discussed below.

### Mutagenic method I

Selected fungal strain KV13 was treated with two cycles of \(\gamma\)-irradiation (Fig. 1). The best \(\gamma\)-ray mutant strain X2-2 was then treated with UV irradiation. The best mutant UV-12 strain was sequentially treated with four cycles of NTG. Finally, mutant strain XN15

### Table 3. Improvement of the production of RSDE by treatment with various mutagenic agents.

<table>
<thead>
<tr>
<th>Mutagenic treatment</th>
<th>Selected mutant</th>
<th>Enzyme production improved (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>KV-13</td>
<td>100*</td>
</tr>
<tr>
<td>1(^{st}) (\gamma)-ray</td>
<td>X-2</td>
<td>120.50</td>
</tr>
<tr>
<td>2(^{nd}) (\gamma)-ray</td>
<td>X2-2</td>
<td>140.00</td>
</tr>
<tr>
<td>UV</td>
<td>UV-12</td>
<td>142.50</td>
</tr>
<tr>
<td>1(^{st}) NTG</td>
<td>N-1</td>
<td>160.21</td>
</tr>
<tr>
<td>2(^{nd}) NTG</td>
<td>N-2</td>
<td>169.56</td>
</tr>
<tr>
<td>3(^{rd}) NTG</td>
<td>N-3</td>
<td>182.20</td>
</tr>
<tr>
<td>4(^{th}) NTG</td>
<td>XN-15</td>
<td>200.70*b</td>
</tr>
</tbody>
</table>

Statistical analysis \((P) <0.0001\)

The 100% enzyme activity was 30.25 U/ml.

The 200.70% enzyme activity was 60.71 U/ml.
produced the highest activities of RSDE, and was selected (Fig. 1 and Table 3). The mutant strain XN15 showed a 200.7% improvement in activity when compared with a wild variety. The mutant XN15 was identified as *Aspergillus* sp. with 99% of homology by sequencing the internal transcribed spacers.

**Mutagenic method II.** Fungal strain X2-2 obtained from method I was simultaneously treated with NTG and UV. After treatment, the best mutant NU-1 was again simultaneously treated with NTG and UV (Fig. 2). As a result, the best mutant showed 145.35% improved enzyme production than that of a wild type. However, further treatment of a 2nd NTG simultaneously combined with UV suddenly decreased the enzyme production from 142.35% to 112.37% (Table 3). Based on the results of enzyme production by mutants created by mutagenic method I and method II, it was concluded that method I was more suitable to make mutant fungi for improvement of RSDE production.

It was reported that when fungi were grown with mutagens in sublethal concentrations, secretive enzyme production increased [9]. Mutant strain XN15, producing a high level of RSDE, was obtained by mutagenic method I. In addition, the strain was stable for a long period while very actively producing RSDE. Previously, the use of mutagenic agents such as γ-ray, UV, and NTG, for the hyperproduction of industrial products was reported; however, the repeated and sequential use of all of these mutagenic agents together in a single study has been undertaken here for the first time. For example, a mutant of *Thermomyces lanuginosus*, obtained after treatment with three cycles of UV and NTG, produced α-amylase and glucoamylase 7- and 3-folds, respectively, higher than those of a wild type [29]. After *F. oxysporum* was treated with UV and then was treated with NTG, the activity of CMCase was increased 3-folds when compared with those of a wild specimen [15]. Mutant *A. niger* after γ-irradiation produced glucoamylase 2.0–2.5 higher than those of wild varieties [27]. Chand et al. [8] used simultaneously NTG, ethidium bromide, and UV, or NTG combined with ethidium bromide to mutate a fungal strain, and the mutant strain secreted more CMCase and filter paper activity than that of wild type. Although the scope of these tests proved interesting, for the purposes of the present paper, we used sequential treatments of three mutagenic agents (γ-ray, UV, and NTG) with the goal of hyperproduction of RSDE; mutation method I was used, with treatment of mutagen sequentially at sublethal concentrations, and shown to improve RSDE production in the best mutant strain.

**Stability of the Mutant Aspergillus sp. XN15**

The mutant *Aspergillus* sp. XN15 was very stable in RSDE production during 17 subculturings, and showed consistently high activity, at 60.75±1.15 U/ml, in basal wheat bran over the period tested.

**Optimal SSF Conditions for the Production of RSDE by Mutant Aspergillus sp. XN15**

There are several factors affecting the SSF process, among which optimization of cultivation parameters alongside selection of a suitable substrate, is crucial.

**Substrate**

Among several solid media, wheat bran showed the highest production of RSDE (60.61 U/ml), whereas rice husk, rice bran, and sawdust exhibited lower enzyme productions at 30.55, 52.12, and 2.66 U/ml, respectively. Wheat bran proved to be the most suitable for the colonization of *Aspergillus* sp. XN15, as indicated by the maximum visible growth on medium and high enzyme yields. Hence, solid wheat bran was used for further testing.

**Initial Moisture Content of Medium**

Enzyme production profiles depended on moisture levels. Moistened solid medium containing 50% moisture content yielded the highest levels of RSDE (62.52 U/ml), whereas those containing 20%, 30%, 40%, 60%, 70%, and 80% produced 47.69, 50.25, 53.09, 56.56, 47.95, and 42.15, respectively. Therefore, a moisture level of 50% was used for subsequent studies. In SSF, moisture levels play an important role in biosynthesis and the secretion of enzymes. High moisture results in decreased substrate porosity as well as reduced oxygen penetration [16], but low moisture leads to poor microbial growth and poor accessibility to nutrients [22]. However, the optimal moisture level depends on the particular fungal strain; for example, *Fusarium solani* produced the highest glucoamylase with wheat bran at 70% moisture [6], whereas for *Aspergillus* sp. A3 the highest levels were recorded at 80% moisture [10], and for *R. nigricans* PCSIR18 at 60% moisture [17].

**Culture Temperature**

Temperature is an important factor and strongly affects the SSF process, which has varying results for glucoamylase production dependant on the organism type and even slight changes in temperature [7, 22]. In this study, the optimal temperature for the highest production of RSDE was found to be 30°C (Fig. 3A). It has been reported that optimal growth temperature varied with different fungi such as at 35°C for *A. niger* NRRL 3112 and *A. niger* NRRL 337 but at 30°C for *Aspergillus* sp. A3 [10]. An incubation temperature of 30°C proved to be best for α-amylase production by *A. oryzae*, with higher temperatures harmfully affecting enzyme production [28].

**Initial pH of Medium**

Among the physical parameters, the pH of the growth medium also plays an important role by inducing morphological change in microorganisms, and in enzyme secretion. A high production of RSDE was observed at a
medium pH of 4.5 (Fig. 3B) and the results reported that production of RSDE was influenced by the pH of the medium, which proved very important in the growth of the microorganism and its metabolic activities. The metabolic activities of the microorganisms are very sensitive to changes in pH, and the exact optimal pH varies between different microorganisms and enzymes. Thus, *A. niger* NCIM1245 produced maximum glucoamylase yields at pH 4.7 [24], but *Aspergillus* sp. A3 at pH 5 [10]. In this study, a medium pH of 4.5 medium was used for further studies.

**Culture Time**

The highest levels of production of RSDE (65.72 U/ml) were observed after 3 days of incubation (Fig. 3C). Incubation beyond 3 days proved undesirable as this resulted in a decrease in enzyme production.

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**Fig. 3.** Effects of various culture conditions on the enzyme production. Conditions tested were (A) incubation temperature, (B) pH of solid medium, (C) culture time, (D) aeration area, (E) liquid seed culture age, and (F) inoculum size. The inoculum contained a spores count of $2.1 \times 10^8$/ml.
**Aeration Area**
Differing quantities of moistened wheat bran per flask volume were found to have affected porosity and aeration. It was found that the highest production of RSDE (70.15 U/ml) was observed at the ratio of substrate mass to flask volume of 3:100 (15 g/500 ml flask) (Fig. 3D) and hence this ratio was used for further studies. Similar findings were reported by Bhatti *et al.* [6] and Ellaiah *et al.* [10], where a ratio of substrate mass to flask volume of 1:50 was optimal for glucoamylase production by *Fusarium solani* and *Aspergillus* sp. A3.

**Age of Seeding Culture**
It was found that a seeding culture age of 2 days was suitable for inoculation and enzyme production, but beyond 2 days was undesirable as it resulted in a decreased enzyme yield (Fig. 3E). In the medium inoculated with 2-day-old seeding culture, the highest production of RSDE (75.85 U/ml) was observed.

**Inoculum Size**
Maximum production (77.87 U/ml) was observed at an inoculum size of 25% (v/w) (Fig. 3F). This inoculum size was used for further tests. Inoculum density is an important factor in an SSF process. Too high inoculum levels increase spore concentrations and water content in the solid medium, which prevents oxygen penetration and thereby inhibits fungal growth and enzyme induction. On the other hand, too low inoculum sizes require a longer time for fermentation to finish.

**Effect of Various Medium Additives on Enzyme Production**
From the achieved data of enzyme production (Fig. 4A), it was found that with the single addition of a carbon source such as sucrose, maltose, rice starch, or corn starch into the solid medium, there was a slight enhancement in the enzyme production of *Aspergillus* sp. XN15; but with the addition of glucose to the medium, there was a significant enhancement of enzyme production (83.52 U/ml). In other reports, glucoamylase production by fungi in wheat bran was enhanced by supplementation with sucrose [3], sucrose and glucose [7, 28], fructose [10], and starch [6, 28], but improvement was not observed with maltose supplements [28].

Different additional nitrogen sources also showed an enhancement in enzyme production by the mutant fungus.

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**Fig. 4.** Effects of various medium additives on the enzyme production.
Additives tested were (A) carbon, (B) nitrogen, (C) surfactant, and (D) metal. M. extract, Malt extract; Y. extract, Yeast extract; Trypt, Tryptone; T. Soy, Tryptic soy; NB, Nutrient broth; S. Milk, Skim milk; Cas, Casaminoacids.
It was found that the addition of urea or NH₄NO₃ resulted in the highest RSDE production of 97.85 and 97.50 U/ml respectively, whereas other nitrogen sources showed a lesser enhancement, or an inhibition in enzyme production (Fig. 4B). An addition of nitrogen additives for improvement of enzyme production was also reported, where (NH₄)₂SO₄ enhanced glucoamylase production by A. awamori [3], yeast extract and peptone enhanced glucoamylase production [7, 28], and urea and ammonium salts also enhanced glucoamylase production [6, 10, 28]; however, an inhibition in enzyme production was found in media supplemented with sodium nitrate [28].

The use of surfactants and fatty acids is well documented to increase the production of hydrolytic enzymes [31]. Surfactants have the potential to enhance microbial growth in SSF by promoting the penetration of water into the solid substrate matrix, leading to an increase in surface area [4]. In this study, we found that Tween 80 enhanced most highly the production of RSDE (89.75 U/ml) (Fig. 4C).

The influence of metal ions on fermentative metabolism is even less well documented. Cu²⁺ is an essential metal ion for all organisms [30]. An addition of calcium ions into the medium enhances α-amylase activity [20] and improves glucoamylase activity [3]. In the present study, it was shown that CoSO₄ enhanced most highly RSDE production (88.52 U/ml) (Fig. 4D).

Production of RSDE in Optimal Conditions with a Solid Medium Supplemented with Additives

Optimal culture conditions for solid wheat bran for production of RSDE was accomplished; a culture temperature of 30°C, medium pH of 4.5, medium moisture content of 50%, 2-day-old seeding culture age, inoculum size of 25%, nitrogen additives of 1% urea and 1% NH₄NO₃, carbon additives of 1% glucose, metal additives of 2.5 mM CoSO₄, surfactant additives of 0.05% Tween 80, and substrate mass to Erlenmeyer flask volume of 3:100 (w/v). Under these conditions, the production of RSDE was 581.41 U/ml. The results showed that the production of RSDE by mutant Aspergillus sp. XN15 was improved 19.4 times that of a wild variety produced in pre-optimized conditions with a basal medium and without additives (Fig. 5). The Yᵣᵣ of 0.47 g/g (g ethanol per g substrate utilized) using mutant-derived RSDE of Aspergillus sp. XN15 was higher than that reported by Rajoka et al. [27]. The ethanol fermentation efficiency using Aspergillus sp. XN15 was 92.7–94.0%, which is a very high efficiency of ethanol production. The simultaneous saccharification and fermentation process was carried out without the application of liquefying enzymes cooking and is hence more economically attractive.

It can be concluded that the Aspergillus sp. XN15 mutant is a potential microorganism for the production of RSDE. In addition, the results of the present study

Ethanol Production from Raw Starch Using Crude RSDE of Mutant Aspergillus sp. XN15 by Simultaneous Saccharification and Fermentation

The mutant-derived RSDE Aspergillus sp. XN15 proved excellent to treat raw starch, with a high efficiency of ethanol conversion. The simultaneous saccharification and fermentation using the crude enzyme of Aspergillus sp. XN15 produced 15.82% and 20.75% (v/v) ethanol from 25.73% and 35.78% (w/v) of rice–corn slurry, respectively, after 96 h (Fig. 6). The Yᵣᵣ of 0.47 g/g (g ethanol per g substrate utilized) using mutant-derived RSDE of Aspergillus sp. XN15 was higher than that reported by Rajoka et al. [27]. The ethanol fermentation efficiency using Aspergillus sp. XN15 was 92.7–94.0%, which is a very high efficiency of ethanol production. The simultaneous saccharification and fermentation process was carried out without the application of liquefying enzymes cooking and is hence more economically attractive.

It can be concluded that the Aspergillus sp. XN15 mutant is a potential microorganism for the production of RSDE. In addition, the results of the present study

Fig. 5. Improvement of the production of RSDE by selected Aspergillus sp. after strain improvement and optimization of medium. WT in BM; enzyme production by wild-type strain in basal medium of solid wheat bran. MT in BM; enzyme production by mutant XN15 in basal medium of solid wheat bran. MT in OM; enzyme production by mutant XN15 in optimized medium of solid wheat bran.

Fig. 6. Simultaenous saccharification and fermentation of raw starch slurry. (A) Rice–corn slurry containing 35.78% (w/v) of solid was treated with RSDE of Aspergillus sp. XN15. (B) Rice–corn slurry containing 25.73% (w/v) of solid was treated with RSDE of Aspergillus sp. XN15.
provided valuable information on the novel efficient mutation methods for the strain improvement.

REFERENCES


