

Hyper-production of raw-starch-digesting enzyme by mutant fungal strain and optimisation of solid by-products

Sản xuất cao sản enzyme phân hủy tinh bột sống bởi chủng đột biến và môi trường tối ưu

Research article

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Selected fungal strain for production of raw-starch-digesting enzyme by solid state fermentation was improved by sequential exposures to γ -irradiation of Co⁶⁰, ultraviolet and treatments with N-methyl-N'-nitrosoguanidine. Mutant *Aspergillus* sp. CXN2-3A was chosen and its production of raw-starch-digesting enzyme (RSDE) was improved 2 folds higher than that of wild type. Optimal condition for the production of the enzyme using wheat bran as the substrate was accomplished for the CXN2-3A. With the optimal fermentation condition and the solid medium supplemented with urea and NH₄NO₃, CoSO₄, Tween 80, 1% glucose, CXN2-3A produced RSDE 19.23 folds higher than wild type cultured in pre-optimized condition and un-supplemented medium.

Chủng nấm chọn lọc sản xuất enzyme thủy phân tinh bột bằng cách lên men trạng thái rắn, chủng nấm được cải thiện bằng chiếu xạ tia cực tím, tia Co⁶⁰ và các phương pháp xử lý với N-methyl-N'-nitrosoguanidine. Mutant *Aspergillus* sp. CXN2-3A, đã được lựa chọn để sản xuất enzyme (RSDE) thủy phân tinh bột sống cải thiện cao hơn 2 lần so với chủng dại. Điều kiện tối ưu cho việc sản xuất các enzyme bằng cách sử dụng cám, lúa mì đã được thực hiện cho CXN2-3A. Với điều kiện lên men xộp tối ưu và bổ sung urê và NH₄NO₃, CoSO₄, Tween 80, 1% glucose, CXN2-3A đã sản xuất RSDE cao gấp 19,23 lần so với kiểu dại ở cùng điều kiện.

Keywords: *Aspergillus* sp., N-methyl-N'-nitrosoguanidine, raw-starch-digesting enzyme, ultra violet, γ -irradiation of Co⁶⁰

1. Introduction

Raw-starch-digesting enzyme (RSDE) composed of glucoamylase and other amylases is capable of hydrolyzing raw starch to glucose and other oligosaccharides at mild temperatures without requiring conventional two-step process of liquefaction and saccharification for ethanol fermentation (Kelly *et al.*, 1995; Yamamoto, 1994). The use of RSDE provides more advantages in conversion of biomass into sugars than single enzyme and acid hydrolysis since it gives higher quality products, energy efficiency, safer working environment and last longer equipment (Matsumoto *et al.*, 1982). RSDE was found in fungal strains such as *Aspergillus* sp., *A. usami*, *Rhizopus* sp., (Abe *et al.*, 1988; Abu *et al.*, 2005; Matsumoto *et al.*,

1982). The improvement of microbial strains using different mutagenic agents for the over-production of industrial products has been considered in commercial fermentation process (Parekh *et al.*, 2000; Vu *et al.*, 2010). *Thermomyces lanuginosus* was treated with irradiation of ultraviolet (UV) and N-methyl-N'-nitrosoguanidine (NTG) to improve α -amylase and glucoamylase (Rubinder *et al.*, 2002). *A. niger* was treated with γ -irradiation of Co⁶⁰ for improvement of glucoamylase production (Rajoka *et al.*, 2004). In this study, various mutagenic agents such as γ -ray of Co⁶⁰, UV, and NTG were combined to mutate fungal strains, then optimization of solid culture for hyper-production of RSDE.

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2. Materials and methods

2.1 Strain improvement by mutations

Fungal strain C6.1 was found to be potent fungal strain for RSDE production and this strain was used for the strain improvement by mutation. The spores of C6.1 were harvested from 6 d-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 PDA plate containing 0.1% of raw starch-RBB and then incubated at 30°C for 4 ÷ 7 days. Based upon the clearing zones surrounding the colonies, γ -ray mutant X2 was selected and was again treated with γ -ray of 2 KGy. According to Kuhad *et al.* (1994), the best 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 at 5 min interval the spores were taken out to test. The best mutant strain UV1 was sequentially treated with four cycles of NTG solution (100 μ g/ml in 0.2 M citrate buffer, pH 5) for 10 ÷ 60 min. After treatment with various mutagenic agents, many mutant strains were screened. Finally, one best mutant strain CXN2-3a was taken for further study.

2.2 Determination of RSDE activity

The RSDE activity was determined as described in previous study (Vu *et al.*, 2010). One unit (U) of RSDE activity was defined as amount of enzyme in 1 mL, which liberates one μ mol of glucose, per min, from raw rice starch (Miller, 1959).

2.3 Preparation of seeding culture

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

2.4 Solid state of fermentation (SSF)

A 500 mL Erlenmeyer flask containing 20 g of rice bran with 33.3% (v/w) of moisture was plugged with cotton and sterilized for SSF.

2.5 Optimization of SSF

The SSF was carried out to study the effect of various parameters required for the optimum production of RSDE by selected mutant CXN2-3A. 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, 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), inoculum size (10, 15, 20, 25, 30, 35, 40, 45, and 50%, v/w) were the parameters to be optimized.

The examined additives were carbon sources of glucose, maltose, rice starch, sucrose, and corn, each at 1%; nitrogen sources of urea, yeast extract, tryptone, tryptic soy, peptone, nutrient broth, skim milk, casamino acids, soy-tone, malt extract, NH₄Cl, and NH₄NO₃, each at 1%; metal salts of CaCl₂, MgCl₂, MnCl₂, CuSO₄, CoSO₄, FeSO₄, ZnSO₄, NaNO₃ and KCl, each at 2.5 mM; surfactant of Tween 20, Tween 80, Trixton-X100, each at 0.5% while SDS, and EDTA, each at 0.4 mM. 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 1d-old seeding culture.

2.6 Ethanol production by simultaneous saccharification and fermentation using RSDE and *Saccharomyces cerevisiae*

Uncooked rice winery waste rice-wine-cake slurry containing 25.73% (w/v) solid was used for simultaneous saccharification and fermentation (SSF). The SSF was conducted in a 100 liters fermentor containing 50 L slurry, pH 3.5, with crude enzyme of fungal strain of mutant CXN2-3a with 1.13 Unit of RSDE/ gds (gram dried substrate). To the slurry, yeast cells of *S. cerevisiae* KV25 was inoculated to make initial cell number of 2.5 x 10⁷/mL, incubated at 30°C for 5 days, and the ethanol formed was determined.

3. Results and discussion

3.1 Screening and selection of a mutant strain

Selected fungal strain C6.1 was treated with two cycles of γ -irradiation. The best γ -ray-mutant strain X2-2 was then treated with UV irradiation. The best mutant UV strain was sequentially treated with four cycles of NTG. Finally, mutant strain CXN2-3A produced the highest activities of RSDE was selected. The mutant strain CXN2-3A exhibited 2.7 folds improved activity than that of wild type. The mutant CXN2-3A was identified as *Aspergillus* sp. with 99% of homology by sequencing the internal transcribed spacers.

Mutant strain CXN2-3A produced a high level of RSDE was obtained by mutagenic method and the strain was also stable for a long period to produce RSDE. In the past, the use of mutagenic agents such as γ -ray, UV, and NTG for hyper-production of industrial products has been reported but the repeated and sequential use of all of these mutagenic agents as shown in this study was not documented yet. A mutant *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 wild type (Rubinder *et al.*, 2002). Mutant *A. niger* created by γ -irradiation produced glucoamylase 2.0- 2.5 folds higher than those of wild type (Rajoka *et al.*, 2004). Vu *et al.* (2009) used repeated and sequential mutagenesis to mutate fungal strain, as a result, the mutant strain secreted more RSDE activity than that of wild type.

3.2 Optimal SSF-condition for the production of RSDE by mutant *Aspergillus* sp. CXN2-3A

Substrate: Among several solid media, rice bran showed the highest production of RSDE (62.2 U/g) while rice-husk, and saw dust exhibited lower enzyme production. Of the various solid substrates used in this study, rice bran proved to be suitable for the colonization of *Aspergillus* sp. CXN2-3A as indication by the maximum visible growth on medium and the highest enzyme yield.

Initial moisture content: Moistened solid medium containing 50% moisture content yielded the highest level of RSDE (62.2 U/g), while those containing 20, 30, 40, 60, 70, and 80% produced lower activity. In SSF, moisture level plays an important role in biosynthesis and secretion of the enzymes. High moisture results in decreased substrate porosity as well as reduced oxygen penetration (Vu *et al.*, 2010) but low moisture leads to poor microbial growth and poor accessibility to nutrient (Pandey, 1992). *Aspergillus* sp. A3 at 80% of moisture using wheat bran (Ellaiah *et al.*, 2002), *Rhizopus nigricans* PCSIR18 at 60 % of moisture (Mahmmod *et al.*, 1997).

The optimal temperature for the highest production of RSDE was at 30°C (Table 1). The 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 (Ellaiah *et al.*, 2002). Incubation temperature at 30°C proved to be the best one for α -amylase production by *A. oryzae* but higher temperature affected harmfully the enzyme production (Ramachandran *et al.*, 2004).

Among the physical parameters, the pH of the growth medium plays an important role by inducing morphological change in microorganism and in enzyme secretion. The high production of RSDE was observed at medium-pH of 4.5 (Table 1) and the production of RSDE was influenced by pH, which is very important in growth of microorganism and its metabolic activities. The metabolic activities of the microorganisms are very sensitive to changes in pH and also optimal pH varies with different microorganisms and enzymes. Thus, *A. niger* NCIM1245 produced maximum glucoamylase yields at pH 4.7 (Pandey & Radhakrishnan, 1993) but *Aspergillus* sp. A3 at pH 5 (Ellaiah *et al.*, 2002).

In the growth-related production of enzymes, after 3 days of incubation, the highest production (61.0 U/g) of RSDE was observed (Table 1). Incubation beyond 3 days was undesirable as this resulted in decreased enzyme production.

Aeration area (g solid medium / 250 mL Erlenmeyer flask): Amount of moistened rice bran to flask volume affects the porosity and aeration differently. It was proven that the high production of RSDE (65.0 U/g) was observed at (15 g/500 mL flask), therefore, this ratio was used for further study. Similar findings were reported by Bhatti (2007) and Ellaiah (2002), where 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 the seeding culture age of 2 days was suitable for inoculation and enzyme production but beyond 2 days was undesirable as these results in decreased enzyme yield. As a result, the highest production of RSDE (70.5 U/g) was observed (Table 1).

Inoculum size: The maximum production (78.5 U/g) was observed at inoculum size of 25% (v/w). This inoculum size was used for further study. Inoculum density is an important factor in an SSF process.

Effect of various medium-additives on the enzyme production: From the achieved data of enzyme production (Table 1), it was found that with the single addition of carbon source such as sucrose, maltose, rice starch and corn starch into the solid medium, there was a slight enhancement in the enzyme production of *Aspergillus* sp. CXN2-3A but with the addition of glucose to the medium, there was a significant enhancement of enzyme production (83.52 U/g). In other reports, glucoamylase production by fungi in wheat bran was enhanced by supplementation with sucrose (Anto *et al.*, 2006), fructose (Ellaiah *et al.*, 2002), starch (Bhatti *et al.*, 2007; Ramachandran *et al.*, 2004), but the improvement was not observed in maltose supplement (Ramachandran *et al.*, 2004).

It was found that the addition of urea or NH_4NO_3 resulted in the highest RSDE production of 95.5 and 102.2 U/g, respectively, while other nitrogen sources showed a little enhancement or an inhibition of enzyme production (Table 1). The $(\text{NH}_4)_2\text{SO}_4$ enhanced glucoamylase production by *A. awamori* (Anto *et al.*, 2006), yeast extract and peptone enhanced glucoamylase production (Bertolin *et al.*, 2003; Ramachandran *et al.*, 2004) urea, ammonium salts enhanced glucoamylase production (Bhatti *et al.*, 2007; Ellaiah *et al.*, 2002), (Ramachandran *et al.*, 2004).

The use of surfactants and fatty acids is well documented to increase the production of hydrolytic enzymes (Singh *et al.*, 1991). Surfactants have the potential to enhance microbial growth in SSF by promoting the penetration of water into the solid substrate matrix that leads to an increase in surface area (Asgher *et al.*, 2006). Herein, we found that Tween 80 enhanced the most highly the production of RSDE (85.7 U/g).

It is Cu^{2+} which is an essential metal ion to all organisms (Silva & Williams, 1993). An addition calcium ions onto medium enhanced α -amylase activity (Nirmala & Muralikrishna, 2003), improved glucoamylase activity (Anto *et al.*, 2006). In the present study, it was shown that CoSO_4 enhanced the most highly the RSDE production (88.52 U/g).

Production of RSDE at optimal condition with solid medium supplemented with additives: Optimal culture condition of solid rice bran for production of RSDE was accomplished, which were at 30°C, medium-pH of 4.5, moisture content of 50%, 2 d-old seeding-culture age, inoculum size of 25%, nitrogen additives of 1% urea and 1% NH_4NO_3 , carbon additive of 1% glucose, metal additive of 2.5 mM CoSO_4 , surfactant additive of 0.05%

Tween 80, substrate mass to Erlenmeyer flask volume of 3 : 100 (w/v). With this condition, the production of RSDE was 548.2 U/g. The results showed that the production of RSDE by mutant *Aspergillus* sp. CXN2-3A was improved 19.23 folds higher than that of wild type produced in pre-optimized condition and basal medium

without additives (Table 1). Also this 581.41 U/g of RSDE of *Aspergillus* sp. CXN2-3A was much higher than those of commercial enzyme preparation from *Rhizopus* sp. (102.3 U/g) and *A. usamii* (94.5 U/g).

Table 1. Effect of different factors on the RSDE production

Moisture (%)	20	40	50	60	80				
U/g	47.7	53.99	60.2	56.45	45.0				
Temp. (°C)	20	25	30	35	40	45			
U/g	40	45	53.1	42	37.2	33.1			
pH of medium	3	4	5	6	7	8			
U/g	36.3	60.4	58	53	50	47			
Culture time (day)	2	3	4	5	6				
U/g	20	61	57.83	52.5	43.41				
Solid medium (g)/ Flask (250 mL)	15	20	360	40	50				
U/g	65	62.2	45.5	38.2	37.2				
Seeding age (day)	1	2	3	4					
U/g	65.2	70.5	65.3	42					
Inoculum size (%, v/w)	10	20	30	40	50				
U/g	75	73.2	78.5	68.3	62.5				
Carbon additive	None	glucose	corn- starch	su- crose	malt- ose	rice			
U/g	78.5	81.5	80.3	79.3	13.5	81.3			
Nitrogen additive	None	NH ₄ Cl	Urea	Malt extract	Casein	Yeast extract	Tryp- ton	Tryptic soy	Soy- tone
U/g	78.2	74.5	95.5	78.9	74.4	78.5	78.5	76.5	80.5
Nitrogen additive (continued)	NH₄NO₃	Pep- tone	Skim milk	NB					
U/g	102.2	79.5	776.5	97.5					
Surfactant	None	Tween -20	Tween- 80	Triton X100	EDTA	SDS			
U/g	78.2	81.5	85.5	78.5	98.5	70.2			
Metal ions addi- tives	None	Mg²⁺	Mn²⁺	Cu²⁺	Co²⁺	Fe²⁺	Zn2+	K⁺	Ca²⁺
U/g	78.2	74.5	83.2	85.2	90.3	56.6	557.5	62.1	81.3
Parameter	WT in basM	MT in basM	MT in opt,						
U/g	28.5	58.7	548.2						

WT in basM, wild type of strain produced RSDE in basal medium; MT in basM, Mutant strain produced RSDE in basal medium; MT in opt, Mutant strain produced RSDE in optimized medium.

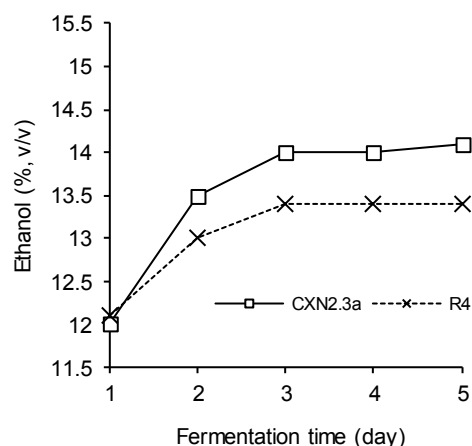


Figure 1. SSF of RWC slurry treated with crude enzyme of fungal strains and *S. cerevisiae* KV-25

SSF of RWC in a 100 L fermentor using crude RSDE of mutant fungal strains of CXN2-3a, together with *S. cerevisiae* KV-25. R4 (commercial enzyme) used as control

Ethanol production from raw starch using crude RSDE of mutant CXN2-3A by SSF: The ethanol production from winery waste rice-wine-cake (RWC) slurry by SSF was conducted in a 100 liters fermentor at 30°C. RSDE from *Aspergillus* sp CXN2-3A was excellent to treat raw-starch with high efficiency of ethanol conversion. The SSF using the enzyme of *Aspergillus* sp. CXN2-3A produced 15.82% (v/v) ethanol from 25.73% of solid, after 96 h (Fig. 1). Also the ethanol fermentation efficiency using

the enzyme of *Aspergillus* sp. CXN2-3A was $92.7 \div 94.0\%$, which is very high efficiency of ethanol production. The SSF process was carried out without cooking and economically more attractive.

4. Conclusion

It can be concluded that fungal strain CXN2-3A mutant is a potential microorganism for production of RSDE. The RSDE produced by the mutant strain can be applied successful ethanol production from slurry containing a high level of raw starch by SSF without application of liquefying enzyme and cooking. On the other hand, the results provided valuable information of using novel efficient mutation method to obtain mutation that produced a high level of RSDE.

5. References

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