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Production and Improving Quality of Intracellular Rennet from *Fusarium subglutinans* Grown in Solid State Cultures

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*F*USARIUM *subglutinans* has a low potency towards production of intracellular rennet substitute in liquid cultures. In this work, solid state fermentation (SSF) technique was found to be suitable for enhancing milk-clotting activity (MCA) of such preparation. The fungus had got the power to produce 19.36 IU/g maize bran when fortified with 0.1% $NH_4(SO_4)_2$, after 7 days under SSF at 30°C. The produced rennet preparation, however, was found to contain unfavourable contaminations of proteolytic (PA) and cellulolytic (CA) activities. Inactivation of these enzyme impurities by heat-treatment at 70°C for 10 min in presence of 0.5% (w/v) NaCl improved to a great extent characteristics of this intracellular rennet where most PA and CA were eliminated at the time that most MCA was preserved.

Keywords: Intracellular rennet, *Fusarium subglutinans*, Solid state.

Production of microbial rennet attracted the interest of many investigators allover the world since the sixties of the last century. This trend of research has been raised due to the progressive decrease of the rennet normally employed in the production of cheese that derived from the abomasums of unweaned calves. Most research works on production of fungal rennet were achieved using the liquid state fermentation either surface or submerged especially those concerned with the extracellular rennets. Two species of the genus *Mucor* were reported to be the most potent sources of this rennet substitute. They are *M. miehei* (Bailey and Siika-aho, 1988; Blatnik *et al.*, 1994; Seker *et al.*, 1998; Beynal *et al.*, 1999; Mariani *et al.*, 2003; De Silveria *et al.*, 2005 and De Lima *et al.*, 2008) and *M. pusillus* (Hegazi, 1983 and Jiao *et al.*, 1992). *Endothia parasitica* (Erdeleyi and Kiss, 1981 and Barkholt, 1987) is also recorded as promising producers. Other fungal genera and species were recorded as good producers in this regard.

Neverthless, fungi and the other microorganisms are rarely reviewed as producers for the intracellular rennet substitutes including those of Menezes and Menezes (1974), Abdel-Rahman *et al.* (1992) and Ghareib *et al.* (2001) are recorded.

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In this work, a trial was performed to enhance the potentiality of *F. subglutinans* as a producer for the intracellular rennet preparation through SSF technique. Quality of the produced enzyme preparation was also tried to be improved.

Matereial and Methods

Organism

F. subglutinans (Wollenweber & Reinking) Nelson *et al.* used in this work was previously isolated from the Egyptian soil and identified by Centraalbureau Voor Schimmelcultures, Netherlands. The organism was grown on Czapek's solid medium at 30°C, maintained at 4°C and monthly subcultured.

Solid state fermentation (SSF) of different brans

Three types of bran i.e. wheat, maize and rice bran were obtained from cereals store in Zagazig, Egypt. Known weight of each bran (5 gm) was added to a 500 ml Erlenmeyer flask and moistened with a little amount of water (10ml) to achieve the SSF conditions .The bran was used simultaneously as nitrogen and carbon source. Different nitrogen sources were also added (in weights equivalent to 0.1 gm $(NH_4)_2SO_4$ / flask) in a subsequent step to test its effect on production of rennet.

The flasks were sterilized at pressure of 15 Lib/inch^{-2}, left to cool, inoculated with spore suspension of 7-day-old culture of the investigated fungus and finally incubated at 30°C. At the end of the incubation period, mixtures of the fungal growth and remainder of the bran were thoroughly washed with 0.2 M phosphate buffer (pH 6) until the washings were free of any of the investigated enzyme activitiy. The washed mixtures were then utilized for extraction of the cell-free extract using 100 ml of the previous buffer as described by Ghareib *et al.* (1988).

Enzyme assays

The intracellular MCA was determined by measuring the time required to clot a reconstituted milk substrate (12% skim milk in 0.01 M $CaCl_2$) as suggested by Kawai and Mukai (1970) and expressed as international units (IU). One IU is defined as the amount of enzyme which clots 10 ml of milk in 1 min under the assay conditions.

Proteolytic activity (PA) was measured by the method of Nomato and Narashi (1959). One unit of protease enzyme was defined as that quantity of enzyme which liberates a product not precipitated by TCA and gave the colour of Folin reagent equivalent to 1μg of tyrosine min^{-1} at 40°C.

Cellulolytic activity (CA) was assayed as exo-β-$(1\rightarrow4)$-glucanase (C_1) by measuring the release of reducing sugar by dinitrosalicylic acid reagent (Miller, 1959). One unit of cellulase activity was defined as the release of one   mole of glucose equivalent min^{-1} at 40°C.

Statistical analysis

Results are expressed as the mean values of three different readings along with standard deviation. T-test and least significant difference (LSD, P > 0.05 = non significant, NS; P: 0.05 to > 0.01 = significant, S and P ≤ 0.01 = high significant. HS) were used in comparison to the control (•) according to the method of Glantz (1992).

Results and Discussion

F. subglutinans was found previously (Ghareib *et al.*, 2001) to produce intracellular MCA at a rate of 0.195 IU/mg dry biomass in submerged liquid cultures. Low yield of the intracellular activity of this organism and all other organisms may be the reason for the little global interest of this type of rennet substitutes.

SSF holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as the enzyme source. This system offers numerous advantages over submerged fermentation system, including high volumetric productivity, relatively higher concentration of the products and less effluent generation and requirement for simple fermentation equipments (Sharma *et al.*, 1991).

SSF was observed suitable for production of extracellular rennet (Jiao *et al.*, 1992; Thakur *et al.*, 1993; Preetha and Poopathy, 1994; Tubesha and Al-Delaimy, 2003 and Da Silveria, 2005), so this technique was tried throughout this work aiming at production of the intracellular MCA of *F. subglutinans* at suitable rates. The results indicate that the fungus had got the power to grow on both wheat and maize bran but not on rice bran. Yields of the intracellular enzyme preparation (Tables 1 a & b) reached 12.3 and 9.81 IU/gm maize and wheat bran after 7 days of SSF, respectively.

TABLE 1a. Solid-state fermentation of intracellular MC enzyme of *F. subglutinans* grown on wheat bran medium.

Incubation period (days)	MCA (IU/g bran)	PA (IU/g bran)	R-factor
2	1.41 ± 0.00 – H	4.87 ± 0.31 –H	0.29 ± 0.02 –H
3	2.00 ± 0.02 – H	6.53 ± 0.61 –S	0.31 ± 0.03 –H
4	2.98 ± 0.05 – H	7.53 ± 0.50 NS	0.40 ± 0.03 –H
5	4.39 ± 0.06 – H	8.73 ± 0.81 NS	0.50 ± 0.05 –H
6	6.82 ± 0.16 –H	9.33 ± 0.11 –H	0.73 ± 0.09 - S
7•	9.81 ± 0.28	8.87 ± 0.81	1.11 ± 0.10
8	8.09 ± 0.34 –H	7.40 ± 0.72 NS	1.09 ± 0.11 NS
9	5.79 ± 0.15 –H	6.20 ± 0.60 –H	0.93 ± 0.09 NS

MCA: Milk Clotting Activity
PA : Proteolytic Activity
R-factor is the ratio of MCA / PA

TABLE 1b. Solid-state fermentation of intracellular MC enzyme of *F. subglutinans* grown on maize bran medium.

Incubation period (days)	MCA (IU/g bran)	PA (IU/g bran)	R-factor
2	1.91 ± 0.14 – H	3.20 ± 0.40 –H	0.60 ± 0.08 –H
3	2.83 ± 0.08 – H	4.13 ± 0.12 –H	0.69 ± 0.02 –H
4	3.61 ± 0.07 – H	6.33 ± 0.31 -H	0.57 ± 0.03 –H
5	4.80 ± 0.14 – H	7.67 ± 0.50 -H	0.63 ± 0.04 –H
6	8.35 ± 0.26 –H	9.53 ± 0.61 NS	0.88 ± 0.06 –S
7•	12.30 ± 0.57	10.60 ± 0.72	1.16 ± 0.08
8	9.99 ± 0.46 –H	10.13 ± 0.42 NS	0.99 ± 0.04 -S
9	7.21 ± 0.24 –H	8.67 ± 0.70 –S	0.83 ± 0.07 -H

In order to improve the enzyme production, different nitrogen sources were added to the more suitable bran-containing medium (maize medium), , in a trial to optimize the conditions required for production of relatively high milk clotting enzyme (MCE) yield. The results (Table 2) demonstrate that addition of $(NH_4)_2SO_4$ to the maize bran medium enhanced production of the intracellular MCA of *F. subglutinans* to reach 16.72 IU/g bran with R-factor of 2.28 IU/PU/g bran (R-factor MCA/PA must be taken into consideration as an important index in production of MCE, Ghareib *et al.*, 2001). Different concentrations of $(NH_4)_2SO_4$ were separately added to the maize bran-containing media (Table 3). The results show that the highest production was obtained when the nitrogen source was added at a level of 0.1 gm /flask.

TABLE 2. Effect of different nitrogen sources on the production of intracellular MCE by *F. subglutinans* grown on maize bran medium.

Added nitrogen sources	MCA (IU/g bran)	PA (IU/g bran)	R-factor
Control•	12.22 ± 0.001	10.42 ± 0.009	1.17 ± 0.013
$(NH_4)_2SO_4$	16.72 ± 0.004 + H	7.32 ± 0.003 – H	2.28 ± 0.043 + H
NH_4Cl	13.07 ± 0.004 + H	12.30 ± 0.003 + H	1.06 ± 0.029 – H
NH_4NO_3	11.10 ± 0.003 – H	10.50 ± 0.004 + H	1.06 ± 0.026 – H
$NaNO_3$	8.70 ± 0.001 – H	5.20 ± 0.008 – H	1.67 ± 0.015 + H
Urea	6.20 ± 0.002 – H	5.10 ± 0.007 – H	1.22 ± 0.030 N.S
Asparagin	5.82 ± 0.002 – H	6.52 ± 0.006 – H	0.89 ± 0.024 – H
Peptone	9.50 ± 0.004 – H	7.30 ± 0.002 – H	1.30 ± 0.055 + S

TABLE 3. Effect of different concentrations of $(NH_4)_2SO_4$ on the production of intracellular MCE of *F. subglutinans* grown on maize bran medium.

$(NH_4)_2SO_4$ concentrations (g/flask)	MCA (IU/g bran)	PA (IU/g bran)	R-factor
0.050	11.30 ± 0.001 – H	5.40 ± 0.008 – H	2.09 ± 0.011 – H
0.075	14.20 ± 0.001 – H	6.12 ± 0.002 – H	2.32 ± 0.004 NS
0.100•	16.24 ± 0.004	7.00 ± 0.007	2.32 ± 0.037
0.125	12.90 ± 0.006 – H	6.82 ± 0.004 – H	1.89 ± 0.050 – H
0.150	9.50 ± 0.003 – H	5.92 ± 0.009 – H	1.60 ± 0.009 – H

The next step was planned to study the influence of changing Solid: Liquid ratio at the time that the bran weight was fixed. Table 4 reveals that the intracellular MCA attained its maximum amounting to 19.36 IU/g maize bran with R-factor of 2.82 when the ratio of solid: liquid was 1:1.5 (w/v). Weight of bran and volumes of water were then changed at the time that their ratios were fixed at the previous optimum level. Table 5 shows that highest accumulation of the intracellular MCA of *F. subglutinans* was obtained when 5 g maize bran fortified with 0.1 gm $(NH_4)_2SO_4$ and moistened with 7.5 ml of water was used for SSF. These results throw the light on the suitability of SSF for production of the present intracellular MCA and these results support those of Tubesha and Al-Delaimy (2003) who recommended the SSF as a very convenient technique for the production of MCE on large scale in industries.

TABLE 4. Solid-state fermentation of intracellular enzyme by *F. subglutinans* as a function of changing solid/liquid ratio on maize bran medium.

Solid : liquid (w/v)	MCA (IU/g bran)	PA (IU/g bran)	R-factor
1 : 1	13.99 ± 0.050 – S	5.00 ± 0.53 – S	2.79 ± 0.09 NS
1 : 1.5•	19.36 ± 0.42	6.87 ± 0.50	2.82 ± 0.08
1 : 2	16.18 ± 0.61 – H	7.00 ± 0.50 NS	2.31 ± 0.050 – H
1 : 2.5	12.29 ± 0.31 NS	6.53 ± 0.42 NS	1.88 ± 0.03 – H
1 : 3	10.25 ± 0.20 – H	5.47 ± 0.42 – S	1.87 ± 0.03 – H

TABLE 5. Solid-state fermentation of intracellular enzyme by *F. subglutinans* as a function of changing weights and volumes at ratio 1: 1.5 on maize bran medium.

Weight : volume (g bran /ml)	MCA (IU/g bran)	PA (IU/g bran)	R-factor
1 : 1.5	9.20 ± 0.00 – H	4.00 ± 2.65 NS	2.30 ± 0.01 – H
2 : 3	12.76 ± 0.16 – S	5.50 ± 1.50 NS	2.32 ± 0.02 – H
3 : 4.5	14.76 ± 0.79 NS	6.11 ± 0.38 NS	2.42 ± 0.03 –H
4 : 6	16.71 ± 1.04 – H	6.51 ± 0.63 NS	2.57 ± 0.03 – H
5 : 7.5•	19.36 ± 0.47	6.85 ± 0.81	2.83 ± 0.08
6 : 9	16.55 ± 0.48 – S	6.22 ± 0.59 NS	2.66 ± 0.22 NS
7 : 10.5	11.21 ± 0.36 – H	4.52 ± 0.44 – H	2.48 ± 0.14 NS
8 : 12	8.62 ± 0.15 – H	3.54 ± 0.07 – H	2.43 ± 0.47 NS

It is worthy to note that the produced intracellular rennet preparation was found to contain impurities of both PA and CA, which are unfavourable for the process of cheese-making. It is advised to remove these impurities without involving costly chemical treatments (Thakur *et al.*, 1993). It was suggested to perform heat-treatment at the highest possible temperatures for the longest period of time, unless the MCA is disadvantageously reduced. The rennet was subjected to heat-treatment at temperatures of 50-80; ¡C. The data (Table 6) reveal that the intracellular MCA of *F. subglutinans* was more resistant to heat-treatment than both PA and CA. Furthermore, the CA was more sensitive than the PA. Heat-treatment for 10 min at 70□C was found to be the most suitable one for elimination of the highest content of CA (58% of the original activity has been eliminated) with 88.1% of MCA was retained.

TABLE 6. Improving quality of the produced rennet by heat treatment.

Temperature (°C)	Time (min)	Remaining intracellular activity (%)		
		MCA	PA	CA
50	1	100.00	100.00	100.00
	3	100.00	99.00	99.30
	5	100.00	97.00	95.20
	7	99.93	94.34	90.10
	10	98.70	90.10	82.64
60	1	100.00	99.60	100.00
	3	100.00	94.95	99.30
	5	100.00	90.00	95.20
	7	99.00	87.10	90.10
	10	97.25	83.20	82.64
70	1	100.00	89.00	97.10
	3	99.00	90.00	88.25
	5	96.18	82.10	81.16
	7	93.00	77.00	67.00
	10	88.10	70.16	42.00
80	1	98.00	96.46	94.00
	3	87.90	81.00	72.55
	5	79.40	69.10	50.00
	7	58.28	43.00	31.18
	10	49.80	20.90	17.00

Although these encouraging results, it was decided to search for additional treatment leading to activation of MCA (or at least preservation of its activity) and inhibition of the remaining PA and CA to obtain enzyme preparation more suitable to be used in cheese-making. Heat-treatment at 70 C was performed in presence of different concentrations of NaCl for different periods. The results (Table 7) revealed that 88.9% of *F. subglutinans* intracellular MCA was retained after 10 min of heating at 70 C in presence of 0.5% (w/v) NaCl. Worthy of mentioning is that CA was greatly inhibited where it is only retained about 22.1%

of its initial activity. Similar results were obtained by Kokusho *et al.* (1976) using heat-treatment in presence of 5% NaCl.

TABLE 7. Improving quality of the produced rennet by combined treatment with temperature and NaCl.

NaCl conc. (%)	Time (min.)	Remaining intracellular activity (%)		
		MCA	PA	CA
0.1	1	100.00	99.55	99.00
	5	98.02	73 11	69.18
	10	94.15	49.93	36.13
0.5	1	100 00	96.14	94.01
	5	97.01	67 11	63.00
	10	88.90	40.02	22.16
1	1	87.27	83.56	84.72
	5	79.61	56.32	50.92
	10	64.99	32.60	17.35
2	1	82.16	65.46	61.70
	5	59.29	41.29	35.18
	10	48.18	28.92	10.82

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إنتاج وتحسين مواصفات الإنزيم المخثر للبن المنتج داخل الخلايا
فى مزارع الحالة الصلبة للفطرة فيوزاريوم سبجلوتينانز

محمد غريب ، حسام حمدى ، آمال خليل وهالة سعد الدين
قسم العلوم البيولوجية والجيولوجية ــ كلية التربية ــ جامعة عين شمس ــ القاهرة
ــ مصر .

وجد من قبل أن الفطرة فيوزاريوم سبجلوتينانز لا تمتلك سوى مقدرة ضعيفة على
إنتاج بديل المنفحة داخل خلاياها فى المزارع السائلة، وفى هذه الدراسة تبين
ملائمة مزارع الحالة الصلبة لتحسين إنتاجية تلك الفطرة موضوع
الدراسة حيث تمكنت للفطرة من إنتاج ١٩,٣٦ وحدة دولية من الإنزيم لكل جم من
ردة الذرة المزودة بـ ١, ٪ من كبريتات الأمونيوم بعد ٧ أيام من تحضين
المزرعة الصلبة عند ٣٠ درجة منوية. وقد وجد أن التحضير الإنزيمى المنتج
يحتوى على كميات غير مرغوبة من إنزيمات البروتييز والسليوليز، وقد تم
التخلص من معظمها بالمعاملة الحرارية عند درجة ٧٠ منوية لمدة ١٠ دقائق فى
وجود ٥, ٪ من كلوريد الصوديوم فى الوقت الذى تم فيه الحفاظ على معظم
النشاط المخثر للبن مما حسن من مواصفات المنتج النهائى.

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