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Influence of Different Salinity Levels and Immobilization on Glycerol Production by Halotolerant Yeast *Zygosaccharomyces rouxii*

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Abstract: Three nitrogen sources, peptone and tryptone as organic sources, and ammonium sulphate as inorganic source for a different incubation periods (72, 84 or 96 hours) at pH 10 or 6.8, at different salinity levels were used. The maximum productivity for total, external and internal glycerol was found in case of using peptone, pH 10 at 14% (w/v) NaCl after 96hs. incubation period (698, 220, 478 mg/L) respectively. Immobilization enhance yeast growth and glycerol production. The productivity of Nylon immobilized cells was higher than immobilized cells on glass wool.

Key words: Halotolerant yeast, glycerol, pH,salinity levels, immobilization

Introduction

Addition of high concentration of NaCl to the growth medium results in a marked stimulation of glycerol production by yeast (Nishi and Yagi, 1993, 1995; Marechal *et al.*, 1995; Ohshiro and Yagi, 1996). In most organisms that can grow at high osmotic pressure, low molecular mass substances are accumulated internally to equilibrate the cytoplasm osmotically with the surrounding media. Adjustment of the internal osmotic pressure with organic solute has been reported for a wide range of microorganisms including halophytic bacteria (Csonka, 1989), fungi (Gadd *et al.*, 1984; Jennings, 1984; Clipson and Jennings, 1990), algae (Ben-Amotz and Averon, 1983), yeast (Van-eck *et al.*, 1989; Jovall *et al.*, 1990, Remize *et al.*, 1999).

The effect of hypertonic concentration of NaCl has been attributed to the formation of glycerol as osmotic agent to facilitate the retention of cellular water (and Brown, 1987) and maintain enzyme activity (Borowitzka and Brown, 1974). Several benefits for the production of biotechnological products can be realized through the use of immobilized systems. The adsorption is process which mainly suitable for viable cells. Microbial cells immobilized by adsorption represents a well established and old method which has been used over many years (Klein and Ziehr, 1990; El-Sayed, 1992; Rehm and Omar, 1993; Dicosmo *et al.*, 1994; El-Batal and Shihab, 1996).

This experiment describe the optimum conditions for glycerol production and effect of immobilization on the production.

Materials and Methods

Microorganism: Locally isolate *zygosaccharomyces rouxii* isolated from marine water of Ismalia, and identified according to the methods of single-linkage and numerical taxonomy comparing with 6 standard strains, then Vernetzungsdiagramm was computed (Mohamed and Farag, 1994).

Media: I-preservation medium g/L. glucose, 5; peptone, 10; yeast extract, 5; agar, 20. II-glycerol production medium. This medium is composed of four solution.1) Sol. A: 5g glucose in 100 ml bidistilled water (10g for inoculum culture. 2) Sol. B: Biotin, 2μg; Ca-pantothenate, 400 μg; inositol, 2000 μg; nicin, 400 μg; P-amino benzoic acid, 200 μg; pyrodoxin hydrochloride, 400 μg; thiamine hydrochloride 400 μg; riboflavin, 200 μg and cyanocobalamine, 20 μg were dissolved in 10 ml biditstilled water. 3) Sol.C : $MgCl_2$. $6H_2O$, 406.6 mg; $CaCl_2$. $2H_2O$, 147.0 mg; Ferric citrate, 10.05 mg; $MnSO_4$. $1H_2O$, 3.38mg; $ZnSO_4$. $7H_2O$, 4.31mg; $CuSO_4$. $5H_2O$, 0.25mg and Citric acid 6.88 mg were dissolved in 100ml bidistilled water. 4) Sol. D: Nitrogen source (Peptone, tryptone or ammonium sulphate), 1 gram; KH_2PO_4, 0.5g. and Na_2SO_4, 0.28g. were dissolved in 790 ml of bidistilled water., pH 6.8 or 10. Different sodium chloride concentrations (w/v) were added to sol. B and D and autoclaved, while sol. A and C autoclaved separately. Then sol. A and C were added to sol. B and D. (Adler and Gustafsson, 1980).

Glycerol Production: Pre-adapted inoculum (the selected strain inoculated into glycerol production medium containing 13.5 g/L NaCl and incubated for 72 hours at 30^{0}C in a shaking incubator) was inoculated (10% v/v) into glycerol production medium containing $(NH_4)_2$ SO_4; Peptone or Tryptone as a nitrogen sources at different salinity levels (10; 12; 14; 16; 18 and 20% w/v NaCl). The pH was adjusted to 6.8 or 10 and they incubated for 72, 84 or 96h.

Immobilization technique: The glass wool (10.0 μm diameter) were added to 250 ml Erlenmeyer flask (0.5 g/flask) containing 60ml of glycerol production medium. After sterilization, they inoculated with 10% (v/v) pre-adapted inoculum and incubated for 96h at 30^{0}C in a shaking incubator.

Optical density of all cultures were determined at 610 nm. Cell dry weights were obtained by drying at 80^{0}C to a constant weight.

Glycerol assay: The grown cultures (10 ml) were centrifuged for 15 min. at 5000 rpm and the supernatant was used as external glycerol. The remainder of the culture were exposed to boiling for 10min., leave to cod and then ultra-sonicated for 10 min. After sonication, the cultures were centrifuged for 15 min at 8000 rpm. The supernatant were used as total glycerol. Enzymatic determination of glycerol using enzyme combination kits (Biochemical test combination, Boehringer Mannheim, Gmb H, Germany).

Glucose assay : Enzymatic determination of glucose using CARO, diagnostic research and marketing, Gmb H, Germany.

Results

The effect of using peptone as a nitrogen source and adjusting pH to 10, incubating the culture for 72h. at different salinity levels on glycerol production was indicated in Table 1. The results showed that as the salinity levels increased, the optical density (O.D) decreased and dry weight (D.wt) also decreased. The total glycerol increased as salinity increased till it reached the maxi.productivity at 14% NaCl, then decreased.

Table 1: Effect of using peptone, pH 10 and incubation for 72h. at different salinity on glycerol production

NaCl	O. D.	D. Wt g/L.	Total Mg/L.	Ext. Mg/L.	Int. Mg/L.	Residual Glucose Mg/dl
10 %	1.891	2.57	522	360	162	0
12 %	1.808	2.36	540	272	278	0
14 %	1.677	2.19	665	300	365	58
16 %	0.724	1.09	289	140	149	75
18 %	0.573	0.98	238	122	116	63
20 %	0.503	0.89	120	50	70	87

Table 2: Effect of using peptone, pH 10 and incubation for 84h. at different salinity on glycerol production

NaCl	O. D.	D. Wt g/L.	Total Mg/L.	Ext. Mg/L.	Int. Mg/L.	Residual Glucose Mg/dl
10%	1.901	2.77	480	160	320	0
12%	1.827	2.37	500	192	308	0
14%	1.727	2.30	680	300	380	7.6
16%	0.742	1.11	320	160	160	70
18%	0.612	1.03	240	96	144	91
20%	0.518	0.93	-	-	-	-

Table 3: Effect of peptone, pH 10 and incubation for 96h. at different salinity on glycerol production

NaCl	O. D.	D. Wt g/L.	Total Mg/L.	Ext. Mg/L.	Int. Mg/L.	Residual Glucose Mg/dl
10%	1.915	2.80	500	188	312	0
12%	1.857	2.43	500	112	388	0
14%	1.781	2.35	698	220	478	0
16%	0.805	1.20	360	184	176	85
18%	0.618	1.04	262	108	154	87
20%	0.528	0.95	124	58	76	93

Table 4: Effect of peptone, pH 6.8 and incubation for 96h. at different salinity on glycerol production

NaCl	O. D.	D. Wt g/L.	Total Mg/L.	Ext. Mg/L.	Int. Mg/L.	Residual Glucose Mg/dl
10%	1.953	2.89	380	4	376	0
12%	1.887	2.58	380	6	374	0
14%	1.866	2.49	360	12	348	0
16%	1.826	2.37	350	5	344	0
18%	1.236	1.69	500	152	348	28
20%	0.657	1.15	228	92	136	71

Table 5: Effect of ammonium sulphate, pH 10 and incubation for 72h. at different salinity on glycerol production

NaCl	O. D.	D. Wt g/L.	Total Mg/L.	Ext. Mg/L.	Int. Mg/L.	Residual Glucose Mg/dl
10%	1.846	2.41	220	10	210	0
12%	1.608	2.10	350	28	322	0
14%	1.560	1.91	440	108	332	0
16%	0.566	0.98	360	168	192	56
18%	0.530	0.96	260	102	158	63
20%	0.441	0.79	144	98	46	68

While the external glycerol decreased as the salinity level increased.This was accompanied by increase in the internal glycerol production till it reached 14% NaCl,then decreased. The residual glucose was 0 at 10 and 12% NaCl.
As the incubation period increased to be 84 h. as shown in Table (2), the O.D, slightly increased and also the dry weights comparing with that of 72 h. incubation period. Also the total glycerol reached the max. productivity at 14% NaCl. Both external and internal glycerol gives the highest productivity also at 14% NaCl. In general the amount of internal glycerol was higher than that of external glycerol. No residual glucose was found in 10 and 12% NaCl.

Table 6: Effect of ammonium sulphate, pH 10 and incubation for 96h. at different salinity on glycerol production

NaCl	O. D.	D. Wt g/L.	Total Mg/L.	Ext. Mg/L.	Int. Mg/L.	Residual Glucose Mg/dl
10%	1.876	2.55	300	28	272	0
12%	1.619	2.18	360	20	340	0
14%	1.596	2.04	460	108	352	0
16%	1.079	1.60	376	112	264	0
18%	0.585	0.98	280	128	152	57
20%	0.509	0.89	160	108	52	60

Table 7: Effect of tryptone pH 10 and incubation for 72h. at different salinity on glycerol production

NaCl	O. D.	D. Wt g/L.	Total Mg/L.	Ext. Mg/L.	Int. Mg/L.	Residual Glucose Mg/dl
10%	1.860	2.50	450	120	320	0
12%	1.694	2.38	465	124	341	0
14%	1.641	2.11	660	300	360	0
16%	0.787	1.08	340	124	216	54
18%	0.623	1.02	260	104	158	67
20%	0.613	1.00	100	76	24	75

Table 8: Growth and glycerol production by free and Immobilized cells of *Z.rouxii* at different salinity levels, using peptone, pH 10 and incubation for 96h.

NaCl	O. D.	D. Wt g/L.	Total Mg/L.	Ext. Mg/L.	Int. Mg/L.	Residual Glucose Mg/dl
10%	1.639	2.10	430	220	210	0
12%	1.022	1.23	500	140	360	54
14%	0.930	1.17	640	220	420	52
16%	0.779	1.09	398	180	218	46
18%	0.607	1.01	298	142	156	38
20%	0.580	0.96	184	136	108	50
Immobilized on nylon						
10%	1.923	2.78	460	320	140	0
12%	1.822	2.37	592	318	274	44
14%	1.065	1.86	668	272	396	44
16%	1.013	1.41	434	198	236	57
18%	0.963	1.19	358	176	182	34
20%	0.865	1.11	240	60	180	33
Immobilized on glass wool						
10%	1.770	2.55	450	280	170	0
12%	1.753	2.30	580	280	300	28
14%	1.036	1.59	650	220	430	43
16%	0.918	1.20	430	175	265	40
18%	0.993	1.22	315	140	175	43
20%	1.075	1.55	264	115	149	32

As the incubation period increased to be 96h., as shown in Table 3 the same trend was found as in Table 1 and 2 for O.D i.e. as the salinity increased, the O.D and D. wt.decreased.O.D and D.wt. after 96 h was slightly higher than that of 72h and 84h.Also total glycerol reached the max. productivity at 14% NaCl. The same for both external and internal glycerol. Generally in all levels of salinity the internal

glycerol was higher than the external one. No residual glucose had been found till 14% NaCl.

The effect of using peptone as a nitrogen source and incubation for 96 h. in medium with pH 6.8 at different salinity levels had been shown in Table 4. The results indicated that O.D and D.wt. were higher in case of pH 6.8 comparing with that of pH 10, in Table 3. The O.D at 18% NaCl in medium with pH 6.8 was 1.236 while this value at pH 10 was 0.618. This indicating the great influence of pH on the growth of the yeast cells. In this case the max. total glycerol productivity was reached at 18% each, indicating that as the salinity level increased, the productivity increased. At pH 6.8, the amount of external glycerol was small compared with the amount of internal glycerol. No residual glucose had been found at 16% NaCl.

The effect of another nitrogen source (ammonium sulphate) in pH 10 at different salinity levels when incubated for 72h. was indicated in Table 5.

The amount of total glycerol produced in general was less in case of $(NH_4)_2\ SO_4$. The max. productivity was 440 mg/L while in case of peptone it was 665 mg/L. The amounts of external glycerol were less than that of internal glycerol.

As the incubation period increased to be 96h., using the same nitrogen source, $(NH_4)_2SO_4$, in medium with pH 10 at different salinity levels as shown in Table 6, the max. productivity of total, and internal glycerol was found at 14% NaCl. A slight increase in total glycerol productivity was found after 96h. Comparing with that of 72h (460 and 440 gm/L) respectively.

By using tryptone as a nitrogen source and pH10 and incubation for 72h. at different salinity levels, as shown in Table 7. The results indicating that the max. total, external and internal glycerol was found at 14% NaCl (660, 300, 360 mg/L) respectively.

The effect of immobilization on glycerol production was shown in Table 8. The results indicated that immobilization enhanced the growth of the yeast cells, and immobilization enhanced the production of glycerol, slightly, so the free cells produced glycerol less than both the cells immobilized on nylon or glass wool. In all cases, the free cells or the immobilized cells, 14% NaCl gives the max. productivity.

Discussion

Glycerol production is the result of various factors interfered with each other. Among the most important factors are, nitrogen source, pH, incubation period and salinity levels. It was found that as the salinity level increased, the lag phase increased i.e. the yeast cells need more time to reach the max. productivity. The characteristics of growth appeared to be very similar in all treatments except that the lag phase was extended by presence of 1 x 5 M NaCl (Burke and Jennings, 1990). Regardless to solute, there was a longer lag time period to growth as a_w was reduced. So glycerol productivity at different incubation period (72, 84 or 96 h.) were determined. Also nitrogen source influence the productivity greatly. It was found that organic nitrogen sources (Peptone and Tryptone) give higher glycerol productivity than that of the inorganic source (ammonium sulphate). In medium in which the nitrogen source of cells was changed from ammonium to urea during the growth cycle, the resulting transition state induced a marked increase in the osmotolerance of the cells (Larsson and Gustafsson, 1993). pH value plays an important role in glycerol production. Many investigator studied the effect of pH on glycerol production in a wide range of halotolerant yeasts. *Debaryomyces hansenii* when grown in alkaline medium, it was better able to select for K^+ against Na^+ than when it grown in more acid medium (Comerford *et al.*, 1985). *Saccharomyces rouxii* grow much more rapidly and to higher cell population level in the absence of added salt than with 18% (w/v) NaCl at either pH 7 or 4.5 (Franta *et al.*, 1986).

Hootmann *et al.* (1991) used media with alkaline pH 9.5 for production of polyols.

Hosono (1991) has grown yeast cells in medium containing 15% (w/v) NaCl and suspended in 100 mM $NaHCO_3$ - Na_2CO_3 buffer pH 9.0 at 30°C to release intracellular material. The amount of proteins and UV-absorbing materials released by osmotic shock were about one-third the amount liberated by mechanical disrupture or alkaline digestion.

This may explain the higher productivity of glycerol in pH 10. Yeast cells under salt-stress at this pH value may be leakage glycerol and the cells to over come the extremely stressed conditions continue to form glycerol intracellularly to balance the external condition in the medium.

Also inoculation of the production medium with pre-adapted inoculum, which grown in glycerol production medium containing 13.5 g/L. NaCl, enhanced glycerol yield greatly (Blomberg, 1997).

Immobilized cells produced glycerol more than free cells. Cells immobilized on nylon fiber was higher in their productivity than that immobilized on glass wool. Also the growth of the immobilized cells were enhanced. Bisping *et al.*, (1990) and Hootmann *et al.* (1991) reported that the highest glycerol productivity reached in batch culture was with immobilized cells of the osmotolerant yeast *Pichia farinosa*.

So the max. glycerol production was found in case of using peptone as a nitrogen source, pH 10 for 96h incubation period at 14% (w/v) NaCl.

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