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Cell immobilization by Agar-Agar & Agarose for protease production by thermotolerant *Bacillus licheniformis JX*849145

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Abstract

In the present investigation, thermotolerant *Bacillus licheniformis JX*849145 was immobilized with gel matrices such as Agar-agar and Agarose. Agarose is a superficial form of agar-agar which is commonly used in to maintain the porosity for mobilization of genetic material. In the present study various concentration were used of Agar and agarose such as 2, 4, 6, 8, 10% and 1, 2, 3% respectively. At 4% of agar 0.78 U/mg/min highest enzyme activity was noted at 24 hrs of incubation period where as 3% of agarose the 0.69 U/mg/min highest activities was found at 66 hrs of incubation period. Comparatively Agar-agar has taken lower time for enzyme production and agarose has taken more time and less production is occurred as compared to agar-agar gel matrix. Hence agar-agar is found more efficient than the agarose for protease production by cell immobilization.

Keywords: *Bacillus licheniformis*, Protease, Immobilization, Gel Matrixes,

1. Introduction

Recently the immobilization of cell and enzymes has more commonly used in the enzyme production industries [1]. During production of beverages, leather products, food products the free enzymes may occurred difficulties during the process hence the immobilization concept was invented by the scientists [2]. There are so many gel matrixes used in

the industries such as Agar-Agar, Agarose, Polyacrylamide, K-carrageenan etc. among them the cheapest and commercially effective gel matrixes are used in the present investigation.

2. METHODOLOGY

Enzyme production medium:

The overnight incubated bacterial culture (4% v/v) was inoculated in 250 ml Erlenmeyer flask having protease production medium (100ml) containing (g/l): Peptone 5, Mannitol 10, $CaCO_3$ 0.3, NH_4NO_3 5, KH_2PO_4 2.0, $MgSO_4$ 0.5, $ZnSO_4$ 0.36 and pH was adjusted to 9.0 by using 0.1 N NaOH. Incubate the synthetic medium on orbital shaking incubator for 18 h at 45°C with 150 rpm. After incubation cells were separated by centrifugation at 14,000 g for 15 min at 4°C and cell free filtrate (CFF) containing protease was collected for analysis of proteolytic activity and protein contents [3; 4].

Enzyme assay:

Protease activity was measured by using casein as substrate [5]. A mixture of 400 µl casein solution (2 % w/v in 50 mM Phosphate buffer pH 7.0) and 100 µl extracted enzyme was added in each tube and incubated for 10 min at 50°C. The reaction terminated by addition of 1ml trichloroacetic acid (TCA) (10 % v/v). The mixture allowed to centrifuge at 14,000 g for 20 min and 1 ml supernatant was removed carefully. Tyrosine/tryptophan content was determined by using Lowery method. The blank was prepared by adding 1ml of TCA before addition of an enzyme. One unit of protease activity (U) is defined as the amount of enzyme that hydrolyzed casein to liberate one µ mole tyrosine per min under the above assay condition [6].

Determination of Total Protein Content:

The total protein contents of the samples were determined according to the method described by Lowry [7]. The protein standard used was BSA (Merck). Protein standard solution, in the range of 0.5 to 5 mg/ml was prepared in triplicate to obtain a standard curve. Samples (cell-free supernatant) were diluted to 1 ml with distilled water so that the protein content would be within the range of the standards. Alkaline copper sulphate reagent (5 ml) was added to each tube and mixed well. The solutions were kept at room temperature for 10 minutes followed by the addition of 0.5 ml Folin & Ciocalteu's Phenol reagent (Merck) working solution. Each tube was rapidly mixed, and incubated in dark for 30 minutes. Absorbance of the samples was measured spectrophotometrically at 570 nm using UV/Vis spectrophotometer (Systronic- model 119) [7].

Cell growth and cell leakage:

The cell growth in freely suspended cultures and cells leaked from the gel beads were determined as cell dry weight by measuring the optical density at 470 nm. One absorbency unit was equivalent to 0.16 g/l (cell dry weight) [8].

Gel Bid formation process:

Agar-agar: Take 2 % to 10 % of agar-agar in a beaker containing pure distilled water, autoclave the medium at 15lbs (121°C) for 15-20 min, after autoclaving cool the medium under tap water and add the 4% of active bacterial culture in it. Pour the mixture of bacterial strain and gel matrix aseptically in petriplate and wait up to 30 min for solidification. Cut the gel by cutter aseptically and used for the protease production process.

Agarose:

Take 1-3% of agarose in pure distilled water and boil it up to complete to become a transparent medium. Cool the medium and add 4% of active bacterial culture in it. Pour the mixture of bacterial strain and gel matrix aseptically in petriplate and wait up to 30 min for solidification. Cut the gel by cutter aseptically and used for the protease production process.

3. RESULTS AND DISCUSSION

In the present investigation we found that the increasing concentration of gel matrix such as agar-agar and agarose decreases the porosity of the gel matrix and the bacterial strain remains in it as a immobilized form. The formed gel bids of gel matrixes are shown in figure 1.

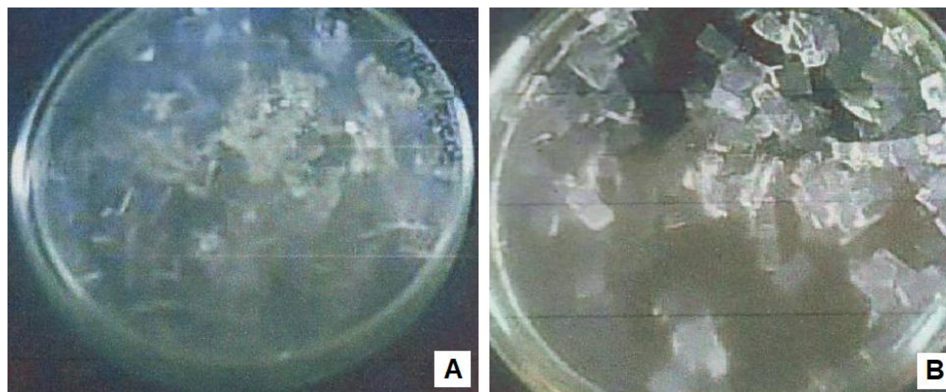

Figure 1: Immobilization of cells in gel matrices (A; Agar-Agar and B: Agarose gel).

Table 1: Protease production by free *B. licheniformis* JX849145 cells used as a control over incubation period of 72 h.

Incubation time (h)	Culture Density (470 nm)	Cells (g/l)	Protease activity (U/mg protein)	Protease activity (%)
0	0	0	0	0
6	0.673	0.107	0.16	13.6
12	1.530	0.244	0.72	61.0
18	2.157	0.345	1.18	100
24	1.531	0.244	0.78	66.0
30	1.489	0.238	0.71	60.1
36	1.460	0.233	0.68	57.6
42	1.486	0.238	0.61	51.7
48	1.496	0.239	0.59	50.0
54	1.389	0.222	0.62	52.5
60	1.402	0.224	0.68	57.6
66	1.468	0.238	0.59	50.0
72	1.423	0.228	0.60	50.8

Protease production by free *B. licheniformis* JX849145:
The extracellular proteolytic activity produced by *B. licheniformis* JX849145 cells (table). It was observed that the amount of cells have profound influence on protease production as productivity was lower with lower amounts of cells. The optimum period of incubation was 18 h after which the cell growth and yield remained the same (Table 1). At 18 h incubation time the protease activity was 1.18 U/mg/min was observed in presence of 0.345 cells (g/l) in the liquid medium. All the obtained results were compared with the free cells of *B. licheniformis* JX849145.

Effect of whole cell immobilization in agar:
The effect of whole cell immobilization in agar on the proteolytic activity of alkaline protease produced from *B. licheniformis* JX849145 was determined by assaying enzyme activity at different concentrations of agar such as 2, 4, 6, 8 and 10% (w/v) and the results of this study are given in Table 4. 29. The maximum enzyme production was observed at different time intervals at different agar concentrations. The obtained results showed at 2% agar the protease activity was 0.58 U/mg/min having 0.332 g/l cell concentration.

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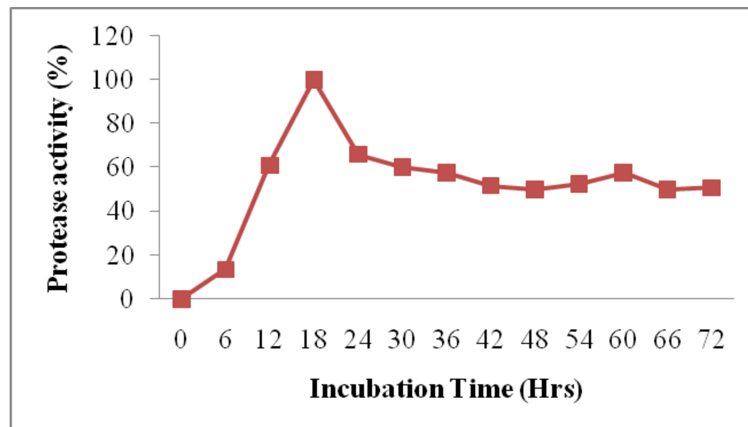

Figure 2: Protease production by free cells

Table 2: Effect of cell immobilization in different concentration of agar on protease activity (U/mg/min) and cell growth

Incubation time (h)	Agar (2%)				Agar (4%)				Agar (6%)				Agar (8%)				Agar (10%)			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0.486	0.078	0.12	20.7	0.423	0.068	0.13	16.7	0.378	0.060	0.09	13.0	0.273	0.043	0.06	9.37	0.198	0.031	0.04	8.70
12	0.569	0.091	0.16	27.6	0.469	0.075	0.15	19.2	0.421	0.067	0.10	14.4	0.290	0.046	0.08	12.5	0.231	0.036	0.06	13.0
18	0.949	0.151	0.23	39.7	0.563	0.090	0.18	23.0	0.598	0.096	0.16	23.1	0.294	0.047	0.08	12.5	0.236	0.038	0.09	19.6
24	0.917	0.146	0.37	63.8	0.860	0.138	0.78	**100**	0.693	0.110	0.15	21.7	0.369	0.059	0.12	18.8	0.256	0.040	0.10	21.7
30	1.463	0.234	0.41	70.7	0.963	0.154	0.59	75.6	0.860	0.138	0.13	18.8	0.393	0.062	0.13	20.3	0.286	0.045	0.12	26.0
36	1.761	0.281	0.49	84.4	1.235	0.198	0.62	79.4	0.891	0.142	0.29	42.0	0.465	0.074	0.27	42.1	0.326	0.052	0.17	37.0
42	2.081	0.332	0.58	**100**	1.584	0.253	0.53	67.9	0.936	0.149	0.45	65.1	0.489	0.078	0.36	56.2	0.356	0.057	0.28	60.8
48	1.986	0.318	0.57	98.2	1.869	0.299	0.70	89.7	1.235	0.197	0.69	**100**	0.569	0.091	0.48	75.0	0.398	0.063	0.32	69.6
54	1.891	0.302	0.56	96.6	1.765	0.282	0.69	88.4	1.365	0.218	0.62	89.8	0.795	0.127	0.64	**100**	0.469	0.075	0.46	**100**
60	1.976	0.316	0.57	98.2	1.968	0.314	0.56	71.8	1.398	0.223	0.59	58.5	0.923	0.147	0.59	92.1	0.635	0.101	0.41	89.1
66	1.823	0.291	0.53	91.3	2.127	0.340	0.63	80.8	1.459	0.233	0.60	86.9	1.263	0.202	0.58	90.6	0.798	0.128	0.40	86.9
72	1.753	0.280	0.46	79.3	1.822	0.291	0.67	85.9	1.365	0.218	0.53	76.8	1.468	0.234	0.59	92.1	1.023	0.163	0.41	89.1

A; Culture Density (470 nm), B; Leaked cells (g/l), C; Protease activity (U/mg/min) and D; Protease activity (%).

Table 3: Effect of whole cell immobilization in agarose on cell growth and protease activity at different incubation time.

Incubation Period (h)	Agarose (1%)				Agarose (2%)				Agarose (3%)			
	A	B	C	D	A	B	C	D	A	B	C	D
0	0	0	0	0	0	0	0	0	0	0	0	0
6	0.563	0.090	0.19	33.9	0.521	0.083	0.17	27.4	0.365	0.058	0.13	18.8
12	0.693	0.110	0.23	40.3	0.536	0.086	0.18	29.0	0.462	0.073	0.15	21.7
18	0.789	0.126	0.27	47.3	0.683	0.109	0.21	33.8	0.532	0.085	0.17	24.6
24	0.896	0.143	0.31	54.3	0.761	0.121	0.27	43.5	0.560	0.089	0.20	28.9
30	0.963	0.154	0.38	66.7	0.860	0.138	0.29	46.8	0.581	0.092	0.20	28.9
36	1.072	0.171	0.42	73.7	0.891	0.142	0.30	48.3	0.630	0.100	0.25	36.2
42	1.236	0.197	0.46	80.7	0.901	0.144	0.37	59.7	0.691	0.110	0.26	37.7
48	1.362	0.217	0.52	91.2	0.963	0.154	0.49	79.0	0.769	0.123	0.38	55.0
54	1.563	0.250	0.57	**100**	0.946	0.151	0.56	90.3	0.986	0.158	0.44	63.8
60	1.632	0.261	0.56	98.2	1.154	0.184	0.62	**100**	1.156	0.184	0.58	84.0
66	1.698	0.271	0.52	91.2	1.198	0.191	0.61	98.3	1.236	0.198	0.69	**100**
72	1.598	0.256	0.54	94.7	1.128	0.180	0.59	95.1	1.189	0.190	0.63	91.3

A; Culture Density (470 nm), B; Leaked cells (g/l), C; Protease activity (U/mg/min) & D; Protease activity (%).

Similarly, at 4%, 6%, 8% and 10% agar concentration the protease activity was 0.78, 0.69, 0.64 and 0.46 U/mg/min respectively. Among all used concentration the 4% agar showed the effective role for production of protease at 24 h. Anna Vassileva [9], reported that the use of agar entrapped cells of *Bacillus circulans* ATCC 21783 for cyclodextrin glucano transferase production in a fluidized bed reactor led to enzyme activity (180 U/ml) after 24 h of cultivation [9]. The cell leakage from the matrix was gradually fluctuated with increases a fermentation time. These findings were in accordance with those obtained previously for the protease production by immobilized *Bacillus* sp. in agar-agar and it was found that the alkaline protease production was started from 6 h onward and reached a maximum level by 24 h (358 U/ml) also observed that the alkaline protease production with immobilized cells in agar-agar was less than the immobilized cells with other matrices [10]. The similar result was observed during Samia et al. [11] investigation as agar (4%) was sufficient for reaching maximal bound enzyme (68.9 U/10ml gel) and immobilization yield (35.2%).

Effect of Whole Cell Immobilization in agarose

The influence of entire cell immobilization in agar on the proteolytic activity of alkaline protease produced from *B. licheniformis* JX849145 was determined by assaying enzyme activity at different concentrations of agarose such as 1%, 2% and 3% (w/v) and the obtained results of present investigation are given in Table 3.

The amount of cell mass entrapped in agarose matrix increased gradually up to 66 h of incubation for all the agarose concentration before and after there was no appreciable change.

4. CONCLUSION

The various gel matrixes are used but cheapest and economically effective gels were used during the present investigation and found that the crude form (Agar-agar) of the gel matrix is more superior than superficial (Agarose) form. The gel matrixes also we can reuse the protease production process hence the occurred difficulties in the various industries this

research may be useful to increase their product yield by reducing the production costs.

Conflict of interest

No conflict of interest influenced in this research.

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