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ORIGINAL ARTICLE

Kinetics of alkaline protease production by *Streptomyces griseoflavus* PTCC1130

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ABSTRACT

Background and Objectives: Proteases are a group of enzymes that catalyze the degradation of proteins resulting in the production of their amino acid constituents. They are the most important group of industrial enzymes which account for about 60% of total enzymes in the market and produced mainly by microorganisms. The attempts were made to study the kinetic parameters of protease produced by *Streptomyces griseoflavus* PTCC1130.

Materials and Methods: *Streptomyces griseoflavus* PTCC1130 was grown on casein agar. Different media such as BM1, BM2, BM3 and BM4 were prepared. Data obtained from growth and protease production were subjected to kinetics evaluation. Casein was used as substrate for protease activity and the released soluble peptide bearing aromatic amino acid were quantified by Folin Cioclateaue reagent. Protein content of the enzyme and the sugar utilized by the organism were estimated by Bradford and Miller's methods respectively.

Results: Basal Medium named as BM1, BM2, BM3 and BM4(50 mL in 250 mL Erlen Meyer flasks) were screened out to evaluate protease production by *Streptomyces griseoflavus* PTCC1130. They were inoculated with known amount of seed culture and kept on rotary shaker. To obtain the specific growth rate, wet weight of biomass was plotted against the time. The clarified supernatant was used for the analysis of protease by measuring the soluble peptide containing aromatic amino acid residues employing Folin Cioclateaue reagent. Our results showed that maximum level of enzyme production (14035 U/L) was occurred at late exponential phase using Basal Medium supplemented with zinc sulfate (0.5g/L), casein (10g/L) at pH 6.5.

Conclusions: A kinetic study of protease production by *Streptomyces griseoflavus* PTCC1130 provided highly quantitative information regarding the behavior of a system, which is essential to study the fermentation process. Exploitation of such kinetics analysis would be useful in commercialization of microbial enzyme production.

Keywords: Kinetics of fermentation, Microbial enzyme, Protease, *Streptomyces griseoflavus*

INTRODUCTION

Proteases are a group of enzymes that catalyze the degradation of proteins resulting in the production of their amino acid constituents (1). They are the most important group of industrial enzymes which account for about 60% of total enzymes in the market (1-3). Alkaline proteases are most widely used in industries. Other than traditional uses in detergent industry for removing protein stains, leather industry for dehairing, textile industry for silk degumming,

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photographic industry for silver recovery, food and feed industry, chemical industry for peptide synthesis, they are increasingly finding potential future applications in pharmaceutical industry too (4). Alkaline proteases are produced by different kinds of microorganisms; bacteria, fungi, yeast, actinomycetes, in addition to its production from plants and mammalian tissues (5-6). The proteases are available in the markets today, are mostly derived from microbial sources. This is due to their high productivity, limited cultivation space requirement, easy genetic manipulation, broad biochemical diversity and desirable characteristics of produced enzyme, such as stability that make them suitable for biotechnological applications (4, 7). Although protease production is an inherent capability of all microorganisms, the microbes that produce a substantial amount of extracellular proteases are of industrial importance (8). Microbial alkaline proteases for industrial uses are produced and studied mainly from *Bacillus* and *Streptomyces* species (3). The possibility of using *Streptomyces* for protease production has been investigated because of their capacity to secrete the proteases into extra cellular media, which can generally be regarded as safe (GRAS)(2). Industrial production of alkaline proteases can be carried out by solid-state and submerged fermentations (4). A kinetic study provides large quantitative information regarding the behavior of a system, knowledge of which is essential to study the fermentation process. In the fermentation process, the metabolism and product production pattern of each microorganism depend mainly on their fermentative, nutritional, physiological, and genetic nature. Exploitation of such microbial metabolism by regulating the critical fermentation parameters helps in economics of commercial production of the required enzyme (8). The production of extracellular protease in microorganisms is greatly influenced produced by the medium components (9-11), metal ions, and physical factors such as pH, temperature, inoculum density, dissolved oxygen and incubation time (9). Also it is known that secretion of the alkaline proteases from actinomycetes is dependent on the growth rate and availability of carbon and nitrogen sources in the medium (12). To the best of our knowledge there is no report on protease production by *Streptomyces griseoflavus*, therefore, in this study a proper culture medium for protease production by the microorganism was screened out. As the growth rate can be influenced by growth conditions, therefore, the relation between specific growth rate and productivity was obtained by studying the fermentation media and initial pH of the media.

MATERIALS AND METHODS

Materials. All the chemicals used for the following experiments were procured from Merck (Germany). 3, 5 Dinitrosalisylic acid was purchased from Sigma Aldrich (USA).

Microorganism and seed culture. *S. griseoflavus* PTCC1130 was obtained from the Persian Type Culture Collection, IROST, Tehran, Iran. The microorganism was grown on nutrient agar slants at 30 °C ±1 and pH 6.5. It was maintained by sub-culturing on nutrient agar slants. For production experiments, the seed culture was prepared by adding a loopful of slants into 50 mLof sterile seed culture medium. After about 24 h (at logarithmic phase), 1.25 mL of seed culture was inoculated into 50 mL of sterile production media.

Identification of proteolytic activity by *S. griseoflavus* **PTCC1130.** Proteolytic activity of *S. griseoflavus* was identified by culturing the organism on casein agar containing zinc sulfate (2) and incubating at 37 °C for 4 days. The medium was then flooded with mercuric chloride solution ($HgCl_2$ 15g, HCl 20 mL and H_2O 80 mL)(13).

Screening of culture media for protease production. For protease production different media were screened, hence four liquid media were prepared as follows: BM1) Basal Medium (containing glucose 0.5 g/L, peptone 10 g/L, KNO_3 0.6 g/L, NaCl 5g/L, K_2HPO_4 0.5 g/L, $MgSO_4$. 7 H_2O 0.5 g/L, $CaCl_2$ 1.0 g/L) (2) and casein 10 g/L was added. BM2) Zn-$SO_4.5H_2O$ 0.5 g/Lwas added to BM1. BM3) It contained BM1 and casein 10 g/L,$ZnSO_4.5H_2O$ 0.5 g/L. BM4) It contained BM1 devoid of casein. The pHs of the media were adjusted at 6.5 and inoculated with 100µl lyophilized culture (spores) of *S. griseoflavus* and were incubated at 30 °C,150 rpm for 168 h, then the cells were separated from the medium by centrifugation and the clarified supernatant was used to determine protease activity. Thereby the media were screened out for highest protease yields.

Protease production. BM3 was used to produce protease (50 mLculture broth in a 250 mL Erlen Meyer flask). The production medium was inoculated with 2.5% v/v of seed culture at its exponential phase (24 h old seed culture). The flasks were incubated on an orbital shaker operating at 150 rpm, 37 °C for a period of 192 h. Samples were withdrawn at the intervals of 24 h and the cells were separated from the culture broth by centrifugation for 30 min at 4 °C , 8000 rpm. Specific growth- rates was determined by plotting wet weight of biomass against time of incubation (2). The clarified supernatant was used for the analysis of protease, total soluble protein and glucose contents. The experiments were carrid out in triplicate and the average values are presented.

Determination of protease activity, biomass, glucose and protein. The protease activity was assayed by incubating casein with protease at 37 ºC for 30 minutes and the reaction was stopped by the addition of 12% trichloroaceticacid (1mL). Thus soluble peptide was measured by Folin Cioclateaue reagent. One unit of protease activity can be defined as the amount of enzyme liberating 1 µM of tyrosine per minute under defined assay conditions (14). The bacterial growth was determined by measuring wet weight which is expressed in terms of g/L(2). The glucose concentration in the fermentation broth was determined by Miller's (15) method using glucose as standard. The total soluble protein of the medium was determined by Bradford method as reported by Whiteley (16), using BSA as standard.

Cell growth kinetics. The exponential growth-phase can be characterized by the following first order equation which states that the rate of increase of cell mass is proportional to the quantity of viable cell mass at any instant time

$dX/dt=\mu X$ (1)

Where dX/dt is the growth rate [g/L h]; X is the concentration of biomass [g/L]; µ is the specific growth rate [h^{-1}](9).

Equation (1) on integration gives

$Ln\ (X-X_0) = \mu t$ (2)

Plot of ln $(X-X_0)$ vs time of incubation, gives specific growth rate (µ) whereat logarithmic phase of microbial growth, $\mu = (\mu)$ max and amount of X_0 for protease production experiments were negligible.

Kinetic analysis. The following kinetic parameters were studied: 1) Maximum specific growth rate (μ_{max}) per hr. The value of μ_{max} was calculated from plot of lnX vs time of fermentation; 2) Product yield coefficient ($Y_{p/x}$ – U/g: the amount of enzyme produced per amount of biomass), its value was determined by the equation: $Y_{p/x} = dp/dx$; 3) Growth yield coefficient ($Y_{x/s}$– g/g: the amount of biomass produced per amount of sugar consumed): Its value was determined by the equation: $Y_{x/s} = dx/ds$; 4) Specific product yield coefficient (q_p- U/g/h: the amount of enzyme produced per amount of biomass per h): the value of q_p was determined by the equation $q_p = Y_{p/x} . \mu_{max}$; 5) Specific growth yield coefficient (q_x- g/g/h: amount of biomass produced per amount of sugars consumed per h): Its value of q_x was determined by the equation:$q_x = Y_{p/x} . \mu_{max}$ (17); and 6) Productivity (P – U/h the amount of enzyme produced per total time of fermentation): Its value was determined by the equation: $P = \Delta P/\Delta t$ (18).

RESULTS

Proteolytic activity of *S. griseoflavus* was proved by culturing the microorganism on casein agar (2) at 37 °C for 4 days. After incubation period was over, the culture medium was flooded with mercuric chloride solution. The clear zone around the colonies (Fig.1) revealed the production of protease (5). Table 1 reveals the screening of the culture media for production of protease by *S. griseoflavus.* As it can be seen from the table, the highest titre of protease activity was obtained in a medium containing casein as an inducer and zinc sulphate as a microelement. Thereby, medium designed as a BM3 was selected to pursue further studies. BM3 was used

Fig. 1. Casein hydrolysis by protease of *S. griseoflavus*

to find out the optimum time required to synthesize protease by *S. griseoflavus*. Both the protease activity and biomass production in accordance with time were taken into consideration. It was observed that the enzyme production was in parallel to biomass production i.e at exponential phase of the growth reaching the maximum at 96 hours incubation at 37 ºC (Fig. 2). Kinetics analysis of protease production in relation to time of incubation, different media and initial pH of the fermentation medium were studied. It was observed that the kinetics parameters were affected by time, half strength medium composition and initial pH (Tables 2, 3, 4). It was found that *S. griseoflavus* produced higher amount of proteaseata bout 96 hours of incubation in a medium designed as BM3 having pH 6.

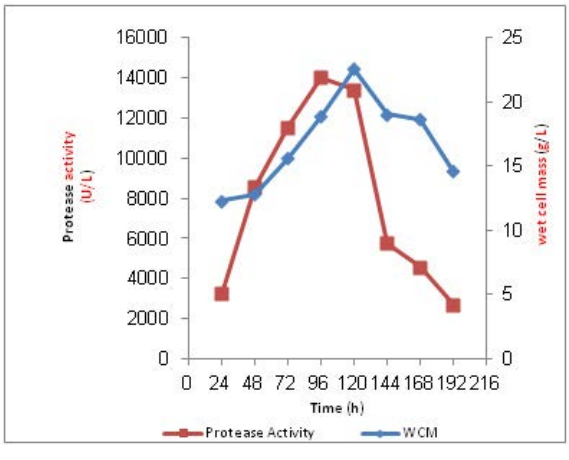

Fig. 2. Effect of incubation time on biomass and protease production by *Streptomyces griseoflavus* at B.M3 medium.

DISCUSSION

Production of protease by culturing *S. griseoflavus* PTCC1130 in an appropriate medium and its growth kinetic in relation to production were studied. Since metalloproteases are generally characterized by a catalytic divalent metal ion such as zinc ion in the active site (4), and according to (Table 1.), addition of zinc sulfate to culture medium greatly enhanced the protease production; it appears that the produced enzyme is a metalloprotease. It was also observed that existence of caseinin culture medium enhanced the amount of protease production by *S. griseoflavus* PTCC1130. This could be due to induction of protease production by casein. The protease production starts at 24 h of incubation, then gradually increased. It exhibited maximum enzyme production (14035 U/L) after 96 h of incubation; while maximum biomass (22.6 g/l) was produced at 120th h (Fig. 2). This is in accordance with the results of Wahid et al. (19), Mostafa Sayed E et al. (20) and Mehta et al. (12) for production of proteases from *Streptomyces bikiniensis, Streptomyces pseudogrisiolus* NRC-15 and an alkaliphilic actinomycete, who reported maximum protease production at the late exponential phase of microbial growth. Also Mukesh Kumar et al. (5) and Rajendran and Thangavelu (9) reported protease production from Bacillus sp. MPTK 712 and *Bacillus sphaericus* MTCC511, during logarithmic phase. This indicates that high level of protease production is found during active biomass production. Culture composition greatly influenced the protease production (Tables 2, 3), productivity (Table 3) and lower maximum specific growth rate μ_{max} which lead to higher productivity (Table 3). In conclusion maximum protease activity could be obtained at initial pH value of 6.5 (Table 4). Both higher and lower pH values led to less protease activity indicating the sensitivity of organism to pH. Except, at pH 6 the lowest μ_{max} was obtained with higher productivity. Thereby a kinetic study of protease production by *S. griseoflavus* PTCC1130 provided fairly good quantitative information regarding the behavior of a system, which is essential to study the fermentation process. Exploitation of such kinetics analysis would be useful in commercialization of microbial enzyme production.

Table 1. Medium cultures used for detection of best medium for protease production

Medium culture	Protease activity (U/L)
B.M1	5700
B.M2	7192.5
B.M3	13375
B.M4	219.25

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Table 2. Effect of incubation time on partial kinetic parameters of protease production by *S. griseoflavus* at pH 6.5

Incubation time (h)	Product yield coefficient ($Y_{p/x}$) (U/g)	Growth yield coefficient (Yx/s) (g/g)	Specific product yield coefficient(q_p) (U/g/h)	Specific growth yield coefficient (q_x)(g/g/h)	Protease activity (U/L)	Specific activity (U/g)	Productivity (U/L /h)
24	264	28.282	38.28	4.100	3255	53010	135.6
48	668	30.614	96.86	4.439	8552.5	84000	178.1
72	737	36.220	106.86	5.251	11535	97750	160.2
96	742	43.209	107.59	6.265	14035	170320	146.1
120	593	51.775	85.98	7.507	13420	237520	111.8
144	301	45.536	43.64	6.602	5745	91190	39.8
168	244	44.678	35.38	6.478	4562	112920	27.1
192	185	34.142	26.82	4.950	2717.5	-	14.1

Table 3. Effect of culture mediumon protease production and partial kinetic parameters of protease production by *S. griseoflavus*

Culture medium	Product yield coefficient($Y_{p/x}$) (U/g)	Growth yield coefficient (Yx/s) (g/g)	Specific activity (U/g)	μ_{max} (1/h)	Maximum protease activity(U/L)	Optimal incubation period (h)	Productivity (U/L/h)
B.M3	742	43.209	170320	0.145	14035	96	146.197
half-strength YEME	57.382	65.64	27142.857	0.0661	1710	120	14.25

Table 4. Effect of initial pH mediumon protease production and partial kinetic parameters of protease production by *S. griseoflavus*

Initial mediumpH	Product yield coefficient($Y_{p/x}$) (U/g)	Growth yield coefficient (Yx/s) (g/g)	μ_{max} (1/h)	Maximum protease activity (U/L)	Optimal incubation period (h)	Productivity (U/L/h)
5.5	127.04	28.81	0.04583	1710	24	71.25
6	127.42	33.092	0.0941	2100	96	21,875
6.5	742	43.209	0.145	14035	96	146.197
7.5	79.11	29.11	0.0639	1140	72	15.833
9	75.39	50.162	0.1139	1403	24	58.458
10	103.54	89.38	0.0994	2980	96	31.041

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