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Original Research Paper

Optimization of medium composition for alkaline protease production by *Marinobacter* sp. GA CAS9 using response surface methodology – A statistical approach

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ABSTRACT

In the present study, the alkaline protease producing *Marinobacter* sp. GA CAS9 was isolated from the marine ascidian *Polyclinum glabrum* and identified by 16S rRNA analysis. Medium components and culture conditions for alkaline protease production were optimized using statistical optimization. Plackett–Burman design was employed to find out the optimal medium constituents and culture conditions to enhance protease production. Central composite design revealed that four independent variables, such as NaCl (60.53 g/l), beef extract (14.73 g/l), $CuSO_4$ (4.73 g/l) and pH (10.7) significantly influenced the protease production. Protease production obtained experimentally coincident with the predicted value and the model was proven to be adequate. The enhancement of protease from 298.34 U/ml to 982.68 U/ml was achieved with the optimization procedure.

1. Introduction

Microbial proteases are important class of enzymes, which constitute more than 65% of the total industrial enzyme market. Alkaline proteases have extensive applications in various industrial such as detergents, food, leather, silk and pharmaceuticals (Mukherjee et al., 2008; Rai and Mukherjee, 2009). Alkaline proteases are produced by a wide range of microbes, including bacteria, molds, yeasts, and mammalian tissues (Mabrouk et al., 1999; Joo et al., 2002). Considering the commercial significance of proteases, there were some attempts to study and maximize protease production and economize them in detergents (Chauhan and Gupta, 2004). For the prospective uses of proteases and their high demand, the need exists for the invention of new strains of marine bacteria that produce enzymes with novel properties and the development of low cost industrial media formulations (Annamalai et al., 2013; Esakkiraj et al., 2011).

Optimization of media components by classical methods which involves the change of single variable optimization strategy has some disadvantages, such as time consuming, requirement of more experimental data sets, and missing the interactions among variables (Cazetta et al., 2007; Li et al., 2008). Owing to these disadvantages, it has been replaced by statistical optimization such as response surface methodology, which is an efficient experimental strategy to seek optimal conditions for the multivariable system. This method has been successfully applied for the optimization of multiple variables in many fermentation processes and showed satisfactory results (Montgomeryd and Runger, 2002).

Marine environment provides various niche that are still pristine and yet to be revealed by their biotechnological potential. One such pristine and diverse group of sessile marine invertebrates is ascidians that serve as host for a range of microbes. Ascidians have a symbiotic relationship with different microorganisms which can be prosperous candidates for protease production (Tatian et al., 2002). In view of that, the present study was aimed to optimize the significant variables such as NaCl, beef extract, $CuSO_4$ and pH on yield of protease production from ascidian associated *Marinobacter* sp. GA CAS9 using Plackett–Burman design and the response surface methodology.

2. Materials and methods

2.1. Ascidian associated bacteria

Marine ascidian *Polyclinum glabrum* was collected from Tuticorin coast, Southeast coast of India by SCUBA divers at a depth of 10–15 m. The ascidan associated bacteria were isolated using Zobell marine agar with Amphotericin B (30 μg/μl) was added to inhibit the fungal contamination and the plates were incubated at 40 °C for 5 days in dark. Isolated colonies were screened for protease production on skim milk agar plates. The bacterial strain GA CAS9 produced the highest clear zone that was considered as

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potential strain and used for further study. The strain GA CAS9 was identified by employing morphological and biochemical characteristics (Holt et al., 1994) and confirmed through molecular characterization. Briefly, the genomic DNA was extracted (Marmur, 1961) and nearly full length 16S rRNA sequences were amplified by using primers 8F (5′-AGA GTTTGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACTT-3′). PCR was performed under the following conditions; 35 cycles consisting of initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and followed by final extension of 5 min at 72 °C. The 16S rRNA gene sequences were obtained by an automated DNA sequencer (Megabace, GE) and homology was analyzed with sequences in the Gene Bank using the CLUSTAL X software. The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). The 16S rRNA gene sequence obtained from the GA CAS9 was deposited into GenBank (NCBI).

2.2. One factor at a time experiments

The media optimization experiment was initiated by enriching in Zobell marine broth supplemented with casein (0.5%) for 3 days at 40 °C. Ten percent of the enriched culture was inoculated in 250 ml flask containing 45 ml basal medium (%w/v) (Casein 1.0, glucose 0.5, K_2HPO_4 0.1, NaCl 3.0, $MgSO_4 \cdot 7\ H_2O$ 0.01 and NH_4NO_3 0.1 at pH 9) and incubated in a shaker (150 rpm) for three days at 40 °C. The cells were harvested by centrifugation at 10,000 rpm for 15 min and the supernatant was further used for protease assay.

Initial screenings of the most significant carbon, nitrogen sources and metal ions to maximize protease production were performed by one-variable-at-a-time approach. Carbon sources such as fructose, lactose, sucrose, xylose, soluble starch and maltose and nitrogen sources such as peptone, skim milk powder, beef extract, meat extract, potassium nitrate, ammonium chloride and sodium nitrate were also individually tested at a concentration of 0.5% in basal medium and various metal ions (zinc sulfate, feric chloride and copper sulfate) were tested for protease production.

2.3. Plackett–Burman design

Plackett–Burman design is an excellent way for screen the main physicochemical parameters from the large number of process variables, which is required for prominent protease production. In the present study, the nutrients were selected based on the results obtained in one variable at time experiments, NaCl, lactose, beef extract, $CuSO_4$ and additionally initial pH, incubation time and incubation temperature were selected to be the major variables in the protease production. The Plackett–Burman method allows evaluation of N variables in $N+1$ experiment, each factor was examined in two levels: -1 for a low level and $+1$ for a high level (Table 1) and the seven variables were evaluated in 12 experimental trials (Table 2). The design was run in a single block and the order of the experiments was fully randomized. The design was developed by the Minitab package version 15.

2.4. Response surface methodology

Based on the selection of the significant variables for protease production by Plackett–Burman design experiment, the significant variables were selected as follows: NaCl, beef extract, $CuSO_4$ and pH. Once the ranges of relevant variables were selected, the response surface methodology, using a central composite design,

Table 1
Variables and test levels for Plackett–Burman experiment.

No	Variables	Levels		Effect	Coefficient	*t*-Value	*p*-Value
		−1	+1				
Constant					373.73	185.21	0.000
X_1	NaCl (g/l)	30	50	−39.43	−19.72	−9.77	0.001
X_2	Lactose (g/l)	5	10	11.93	5.97	2.96	0.042
X_3	Beef extract (g/l)	5	10	72.21	36.11	17.89	0.000
X_4	$CuSo_4$ (g/l)	0.5	1.0	32.93	16.47	8.16	0.001
X_5	Incubation temperature (°C)	30	50	−22.17	−11.09	−5.49	0.005
X_6	pH	7	11	55.86	27.93	13.84	0.000
X_7	Incubation time (h)	40	72	−9.38	−4.69	−2.32	0.081

Table 2
Plackett–Burman design for seven variables with coded values along with the predicted and observed results.

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	**Protease yield (U/ml)**	
								Observed	**Predict**
1	1	−1	1	−1	−1	−1	1	354.018	346.159
2	1	1	−1	1	−1	−1	−1	326.681	328.189
3	−1	1	1	−1	1	−1	−1	384.230	384.728
4	1	−1	1	1	−1	1	−1	440.441	444.328
5	1	1	−1	1	1	−1	1	298.146	296.638
6	1	1	1	−1	1	1	−1	401.654	401.156
7	−1	1	1	1	−1	1	1	487.240	486.315
8	−1	−1	1	1	1	−1	1	391.453	396.350
9	−1	−1	−1	1	1	1	−1	397.234	389.374
10	1	−1	−1	−1	1	1	1	303.162	307.632
11	−1	1	−1	−1	−1	1	1	380.245	381.170
12	−1	−1	−1	−1	−1	−1	−1	320.291	322.755