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Evolutionary Operation (EVOP) to Optimize Whey-Independent Serratiopeptidase Production from *Serratia marcescens* NRRL B-23112

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Serratiopeptidase (SRP), a 50 kDa metalloprotease produced from *Serratia marcescens* species, is a drug with potent anti-inflammatory property. In this study, a powerful statistical design, evolutionary operation (EVOP), was applied to optimize the media composition for SRP production in shake-flask culture of *Serratia marcescens* NRRL B-23112. Initially, factors such as inoculum size, initial pH, carbon source, and organic nitrogen source were optimized using one factor at a time. The most significant medium components affecting the production of SRP were identified as maltose, soybean meal, and K_2HPO_4. The SRP so produced was not found to be dependent on whey protein, but rather was notably induced by most of the organic nitrogen sources used in the study and free from other concomitant protease contaminant, as revealed by protease inhibition study. In addition, experiments were performed using different sets of EVOP design with each factor varied at three levels. The experimental data were analyzed with a standard set of statistical formula. The EVOP-optimized medium, with maltose 4.5%, soybean meal 6.5%, K_2HPO_4 0.8%, and NaCl 0.5% (w/v), gave a SRP production of 7,333 EU/ml, which was 17-fold higher than the unoptimized media. The application of EVOP resulted in significant enhancement of SRP production.

Keywords: Serratiopeptidase, EVOP, *Serratia marcescens* NRRL B-23112, optimization

Serratiopeptidase (SRP) or serrapeptase is a 50 kDa metalloprotease produced by *Serratia marcescens* species [10]. SRP has been gaining wide acceptance in Europe and Asia as a potent analgesic and anti-inflammatory drug [13] and a drug of choice for the chronic inflammatory diseases such as arthritis, fibrocystic breast disease, carpel tunnel syndrome, sinusitis, bronchitis, and atherosclerosis [11]. Recent Japanese patents even suggest that oral SRP may help to treat or prevent viral diseases such as AIDS and hepatitis B and C (Fujisaki *et al*. 1996. JP08040930; Fujisaki *et al*. 1999. JP11199509).

The bacterium *S. marcescens* has been widely reported as a good producer of extracellular metalloprotease. Research on the purification and characterization of SRP from different strains of *S. marcescens*, such as *S. marcescens* E-15 [14–16], *S. marcescens* ATCC 25419 [9], and *S. marcescens* NRRL B-23112 [21], has shown microheterogeneity among the metalloproteases produced. The gene of metalloprotease (SRP) from various *S. marcescens* species has been isolated, cloned, and sequenced [6, 18] and the crystal structure determined [4]. Productivity of SRP by *S. marcescens* is greatly affected by the culture media constituents, especially by the organic nitrogen source. Production medium rich in proteins such as casein, tryptone, peptone, skim milk, and soybean meal extract are excellent for induction of SRP (Isono *et al*. 1972. US Patent No. 3,691,014). Various workers have reported high quantities of protease using these types of media [5, 7, 8, 17]. Some workers report a high level of metalloprotease production only in the presence of whey-enriched media [19, 20]. Ustáriz *et al*. [23] carried out fermentation using individual components of whey to identify the key component responsible for induction of metalloprotease, but not serine protease. Their results showed that the intact whey, but none of its components, could effectively induce metalloprotease. The reason for their observations remained unclear.

Evolutionary operation (EVOP) technique is a powerful statistical tool and is gaining importance in optimization of multiple parameters simultaneously of complex systems like fermentation media [3]. The advantage of the EVOP technique is its simplicity and practicability, being a clear-

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cut decision-making procedure to maximize production. It can be used to optimize simultaneously the effects of two or more parameters to increase microbial metabolite productivity in a laboratory-scale fermentation process [1, 2]. EVOP is actually based on the concept that evolution as a natural process can be significantly accelerated by creating mutations (changing variables), measuring the responses, and then proceeding towards the optimum. The relationship between a response such as yield and the process variables is represented as a three-dimensional "response surface," which in the neighborhood of a maximum or minimum will have the appearance of a mound or a valley, respectively. Results of changes made in a judicious manner are analyzed by statistical principles to see if the variation in response (*i.e.*, yield due to variation in process variable) is due to mere chance (*i.e.*, inherent variations) or due to a significant effect of change in operating variables on the response. When the results from a set of process conditions clearly indicate an advantage due to the changes adopted, these operating conditions become the "new standard." This then becomes the new nucleus for the next phase of EVOP studies in the search for even better optima. Therefore, EVOP is not a short-term or "crash" measure but a continuing program towards achieving the end result [3]. This study reports the optimization of physicochemical fermentation parameters with the help of EVOP design for SRP production without noticeable induction of any other protease contaminant. The yield obtained with the optimized medium is among the highest reported in the literature.

Materials and Methods

Media Components and Microorganism

Glucose, maltose, fructose, sucrose, soluble starch, maltodextrin, glycerol, soybean meal, casein peptone, yeast extract, casein peptone soy meat, soy protein, beef extract, corn steep liquor, peptone bacteriological, proteose peptone, meat peptone, sodium chloride, potassium dihydrogen phosphate, and trichloroacetic acid were purchased from Himedia Ltd., Mumbai, India. Phenylmethylsulfonylfluoride (PMSF) and ethylenediaminetetraacetic acid (EDTA) were purchased from S. D. Fine Chemical Ltd., Mumbai, India. Casein was purchased from Merck Chemicals, Mumbai, India.

S. marcescens NRRL B-23112 was a gift sample from ARS Culture Collection, U.S.A. It was maintained on soybean casein digest agar medium and subcultured after every 15 days.

Effects of Inoculum Size and Initial pH of Fermentation Medium on the Production of SRP

Sterile 5-ml saline solution was added to a well-grown slant tube of *S. marcescens* NRRL B-23112, and gently shaken manually. Then, 0.1 ml of cell suspension having a viable cell count of 1×10^6/ml was transferred to 50 ml of seed culture containing soybean casein digest medium in 250-ml Erlenmeyer flasks. The seed culture was grown for 14 h at 25°C and 180 rpm. The optical density of the seed culture was measured at 660 nm using a spectrophotometer (Helios α, Thermo Electron Corporation, U.S.A.), and it was adjusted to OD of 1.9 to 2.1, which corresponded to a viable cell count of 2×10^8/ml. For optimization of the inoculum size, the inoculum was varied from 0.5 to 12.5% (v/v) of the cells of *S. marcescens* NRRL B-23112 having a viable cell count of 2×10^8/ml (OD 1.9 to 2.1), and transferred to 250-ml Erlenmeyer flasks containing 50 ml of production medium [glucose 0.25, tryptone 2, K_2HPO_4 0.5, NaCl 0.5% (w/v)] and incubated in an orbital shaker at 25°C, 180 rpm for 48 h. All the experiments were performed in triplicates.

The initial pH of the fermentation medium was adjusted to a range of 5.5 to 8.5, inoculated with 1% (v/v) cell suspension (viable cell count of 2×10^8/ml), and incubated in an orbital shaker at 25°C, 180 rpm for 48 h.

Effects of Carbon Sources on the Production of SRP

Various carbon sources including monosaccharides (glucose and fructose), disaccharides (maltose and sucrose), and complex carbon sources (maltodextrin, soluble starch, and glycerol) were screened to find out the most suitable source supporting maximum SRP production. Each carbon source was screened at three different concentrations: 0.25%, 1.00%, and 1.75% (w/v). The other fermentation conditions were 0.5% inoculum, initial pH of 6, and incubation temperature of 25°C at 180 rpm for 48 h.

Effects of Organic Nitrogen Sources on the Production of SRP

A wide range of different protein-rich organic nitrogen sources such as soybean meal, casein peptone, yeast extract, casein peptone, soy meat, soy protein, beef extract, corn steep liquor, peptone bacteriological, proteose peptone, and meat peptone, all at 2% (w/v), replacing the control (tryptone), were screened for maximum SRP production. Other fermentation conditions were 1.75% maltose, 0.5% inoculum, initial pH of 6, and incubation temperature of 25°C at 180 rpm for 48 h.

Optimization of Production of SRP Using EVOP

The methodology involves judicious selection of experimental conditions, obtaining responses by experimentations, and analysis of the data by statistical methods to determine if the responses are significant enough to draw any conclusions. For the study of three-variable systems, the total number of new experiments to be conducted is 2^3, apart from the 2 control (search)-level experiments. The parameters

Table 1. Experimental design for SRP production.

Experiments Parameters (% w/v)	E_{10}	E_{11}	E_{12}	E_{13}	E_{14}	E_{20}	E_{21}	E_{22}	E_{23}	E_{24}
Maltose	0	–	–	+	+	0	+	–	+	–
Soybean meal	0	–	+	–	+	0	+	–	–	+
K_2HPO_4	0	–	+	+	–	0	+	+	–	–
Response (average)	a_{10}	a_{11}	a_{12}	a_{13}	a_{14}	a_{20}	a_{21}	a_{22}	a_{23}	a_{24}

for the EVOP were maintained in both the higher level (+) and lower level (−) compared with the parameters in the search level (0), which is normally assumed to be the initial optimum level. All the real values of each level were calculated from the standard formula, as reported by Tunga *et al*. [22]. Each set of experiments was divided into two blocks (block I and block II) and each of these had one set of control experiments (Table 1). The fermentation was carried out as described earlier with higher and lower levels of maltose, soybean meal, and K_2HPO_4 (Table 5). To minimize standard deviation and error limits, each set was repeated at least twice (cycles I and II). Following the given assay procedure, the enzyme activity of SRP was recorded separately to determine the difference and average values for cycles I and II. Table 2 shows the standard set of formulas used to calculate the effects and error limits of parameters under study for SRP optimization. When the experimental results of the first set did not satisfy the optimum SRP production, a second set of experiments (Set II) was planned (Table 5), selecting the best condition of the first set as the new search level for the second set. This procedure was repeated until the optimum fermentation combination was obtained to maximize the SRP production.

Analytical Determinations

Determination of the SRP activity is based on its caseinolytic property. The method was adapted from Salamone and [21]. To the substrate solution [0.75 ml consisting of 1.0% (w/v) casein in 100 mM Tris/HCl, 1 mM $MgCl_2$, and 2 mM PMSF at pH 8.0], assay sample (0.1 ml) was added and incubation carried out at 40°C. After 30 min, the reaction was quenched with 0.5 ml of 10% (w/v) trichloroacetic acid (TCA) to precipitate the unhydrolyzed casein. After 15 min at 25°C, the reaction mixture was centrifuged at 10,000 ×*g* for 10 min, and the absorbance of the supernatant was determined at 280 nm. One unit of enzyme activity (EU) was defined as the amount of enzyme that produced an increase of absorbance of 0.1 at 280 nm under the conditions of the assay. For the blank, 0.5 ml of 10% (w/v) TCA was mixed with 0.1 ml of assay solution then, 0.75 ml of the substrate solution was added and the mixture allowed to stand for 30 min at 40°C and then the absorbance was determined as described above.

Dry cell weight (DCW) was measured after centrifuging 2 ml of culture broth in a preweighed microcentrifuge tube at 10,000 ×*g* for 15 min, decanting the supernatant, washing the cell pellet twice with distilled water, again centrifuging as above, and drying the cell pellet at 80°C to constant weight.

Protein was determined by the Lowry method using bovine serum albumin as the standard [12].

Inhibition of Protease Activity

The effects of class-specific protease inhibitors 2 mM PMSF and 5 mM EDTA were determined by addition to the standard reaction mixture. The residual caseinolytic activity was determined after incubation at 40°C for 30 min, as previously described.

RESULTS

Effects of Inoculum Size and Initial pH of Fermentation Medium on the Production of SRP

An inoculum size of 1% (2×10^8 cells/ml) supported maximum production of SRP (425±38 EU/ml) and total protein content (4.7±0.2 mg/ml). A gradual decrease in the production of SRP as well as protein content was observed when the inoculum size was increased from 1% to 12.5%. The pH after fermentation and the DCW for each inoculum size remained almost unchanged (Fig. 1).

Table 2. Calculation worksheet of effects of three-variable system, standard deviation, and error limits.

Effect of	Calculation of effects
Maltose	$1/4(a_{13}+a_{14}+a_{21}+a_{23}-a_{11}-a_{12}-a_{22}-a_{24})$
Soybean meal	$1/4(a_{12}+a_{14}+a_{21}+a_{24}-a_{11}-a_{13}-a_{22}-a_{23})$
K_2HPO_4	$1/4(a_{12}+a_{13}+a_{21}+a_{22}-a_{11}-a_{14}-a_{23}-a_{24})$
Maltose×soybean meal	$1/4(a_{11}+a_{14}+a_{21}+a_{22}-a_{12}-a_{13}-a_{23}-a_{24})$
Maltose×K_2HPO_4	$1/4(a_{11}+a_{13}+a_{21}+a_{24}-a_{12}-a_{14}-a_{22}-a_{23})$
Soybean meal×K_2HPO_4	$1/4(a_{11}+a_{12}+a_{21}+a_{23}-a_{13}-a_{14}-a_{22}-a_{24})$
Maltose×soybean meal×K_2HPO_4	$1/4(a_{21}+a_{22}+a_{23}+a_{24}-a_{11}-a_{12}-a_{13}-a_{14})$
Change in mean effect	$1/10(a_{11}+a_{12}+a_{13}+a_{14}+a_{21}+a_{22}+a_{23}+a_{24}-4a_{10}-4a_{20})$
Standard deviation (σ)	$1/2(\sigma_1+\sigma_2)$
σ_1	$R_1\times f$
σ_2	$R_2\times f$
R_1	(largest difference−smallest difference) in block I
R_2	(largest difference−smallest difference) in block II
f	Statistical constant, 0.3
Error limits	
for average	±1.414 σ
for effects	±1.004 σ
for change in mean	±0.891 σ

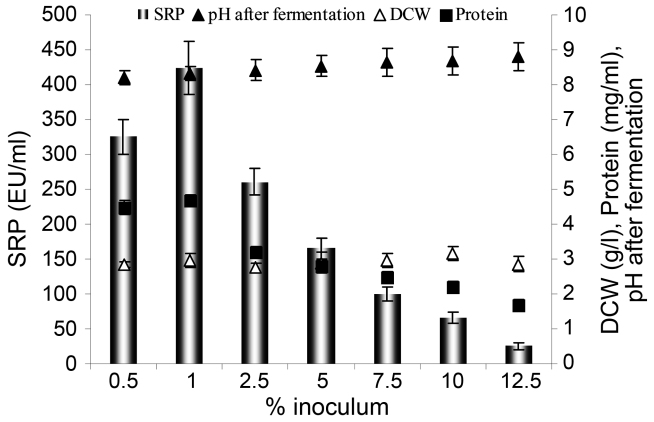

Fig. 1. Effect of inoculum size on the production of SRP.

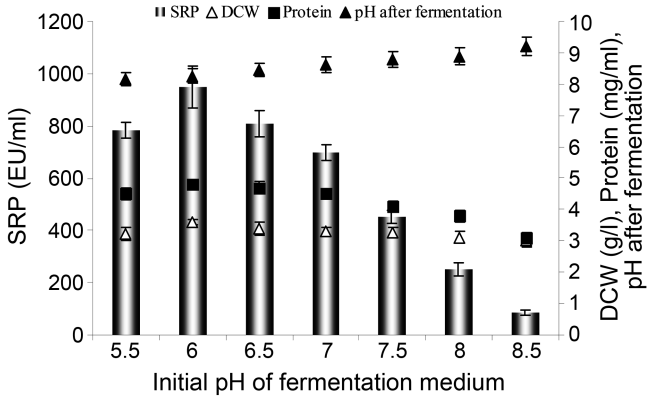

Fig. 2. Effect of initial pH of the fermentation medium on the production of SRP.

An initial pH of 6.0 of the fermentation medium supported maximum SRP production (950±80 EU/ml). As the pH of fermentation increased from 6 to 9, the production of SRP, DCW, and protein content decreased (Fig. 2).

Effects of Carbon Sources on the Production of SRP

The effects of carbon sources (0.25 to 1.75% w/v) on the production of SRP were investigated (Table 3). Maltose, maltodextrin, and glycerol at 1.75% w/v gave enzyme production of 2,650±200, 2,500±210, and 2,400±136 EU/ml, respectively, indicating that they were not significantly different. The production of SRP and protein content increased with an increase in the concentration of all the carbon sources from 0.25 to 1.75% w/v. When 1.75% w/v soluble starch was used as substrate, DCW was maximal at 9.5±0.7 g/l, but the production of SRP was low (2,120±200 EU/ml). All the carbon sources increased the pH of the fermentation broth. Maltodextrin and glycerol gave inconsistent results when checked for reproducibility (results not shown). Hence, further studies were carried out with maltose 1.75% as the carbon source.

Effects of Organic Nitrogen Sources on the Production of SRP

Various organic nitrogen sources (2% w/v) were added to the medium containing 1.75% w/v maltose. The highest

Table 3. Effects of carbon sources on production of SRP.

Carbon sources	Concentration (% w/v)	SRP (EU/ml)[a]	DCW (g/l)[a]	Protein (mg/ml)[a]	pH after fermentation[a]
Glucose	0.25	1,100±40	3.2±0.3	4.8±0.2	8.69±0.2
	1.00	1,450±110	3.0±0.4	6.8±0.3	8.34±0.3
	1.75	1,550±120	2.6±0.2	7.2±0.5	8.12±0.2
Maltose	0.25	1,800±150	4.1±0.4	5.5±0.2	8.68±0.2
	1.00	2,200±160	6.2±0.5	6.3±0.3	8.39±0.3
	1.75	2,650±200	9.1±0.8	8.1±0.2	7.70±0.2
Maltodextrin	0.25	1,820±150	4.3±0.3	6.2±0.2	8.90±0.4
	1.00	2,190±190	6.5±0.5	6.8±0.2	8.74±0.3
	1.75	2,500±210	9.1±0.7	7.3±0.3	8.42±0.1
Glycerol	0.25	1,600±120	4.2±0.3	6.7±0.2	8.69±0.3
	1.00	2,010±125	5.6±0.4	7.2±0.3	8.41±0.2
	1.75	2,400±136	8.5±0.7	7.8±0.4	7.19±0.1
Soluble starch	0.25	1,900±150	4.2±0.3	6.6±0.2	8.76±0.1
	1.00	2,100±200	6.6±0.6	6.8±0.3	8.80±0.2
	1.75	2,120±200	9.5±0.7	7.1±0.4	8.85±0.3
Fructose	0.25	1,600±110	3.2±0.2	4.7±0.2	8.61±0.2
	1.00	1,900±150	4.1±0.2	5.0±0.3	8.10±0.2
	1.75	2,010±190	4.2±0.3	5.1±0.3	7.80±0.2
Sucrose	0.25	1,550±120	2.8±0.1	4.2±0.2	8.42±0.3
	1.0	1,800±180	3.5±0.2	4.8±0.3	8.02±0.2
	1.75	1,950±190	3.9±0.2	5.1±0.2	7.54±0.1

[a]Results are the mean ± SD of three determinations.

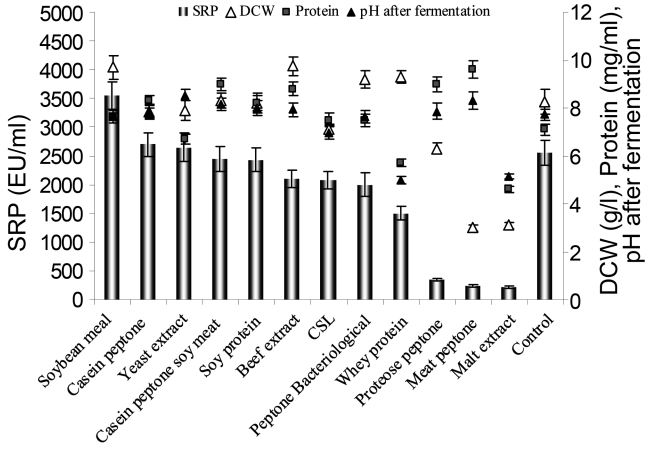

Fig. 3. Effects of organic nitrogen sources on the production of SRP.

production of 3,550±150 EU/ml was obtained with soybean meal, followed by 2,700±210 EU/ml with casein peptone (Fig. 3). All other organic nitrogen sources used in the study produced lower titers of SRP as compared with the control. When beef extract was used as the nitrogen source, DCW was maximal at 9.7±0.4 g/l, but the production of SRP was low at 2,100±160 EU/ml. In the case of proteose peptone and meat peptone, the protein contents were high at 9.0±0.3 and 9.3±0.3 mg/ml, respectively, but production of SRP was poor at 350±20 and 230±19 EU/ml, respectively. An interesting observation was that the pH at the end of fermentation was alkaline with all the nitrogen sources, except for malt extract and whey protein where the pH was acidic. To check the occurrence of serine protease produced along with metalloprotease, inhibition experiments were performed using specific protease inhibitors. EDTA 5 mM inhibited nearly 92–98% of activity and a well-known irreversible inhibitor of serine proteases (PMSF 2 mM) inhibited nearly 1–5% of protease activity with all the organic nitrogen sources used (Table 4). Soybean meal

Table 4. Effects of class-specific inhibitors on the caseinolytic activity of fermentation broth from different organic nitrogen sources.

Organic nitrogen sources (2% w/v)	Inhibitors (mM)	Inhibited activity (%)
Soybean meal	EDTA, 5	92
	PMSF, 2	6
Casein peptone	EDTA, 5	95
	PMSF, 2	3
Yeast extract	EDTA, 5	98
	PMSF, 2	1
Casein peptone soy meat	EDTA, 5	97
	PMSF, 2	4
Soy protein	EDTA, 5	96
	PMSF, 2	5
Beef extract	EDTA, 5	96
	PMSF, 2	3
CSL	EDTA, 5	98
	PMSF, 2	2
Peptone bacteriological	EDTA, 5	97
	PMSF, 2	2
Whey protein	EDTA, 5	98
	PMSF, 2	1
Control (tryptone)	EDTA, 5	95
	PMSF, 2	2

Table 5. Experimental conditions and results of set I and set II.

Set I										
Exp no.	E_{10}	E_{11}	E_{12}	E_{13}	E_{14}	E_{20}	E_{21}	E_{22}	E_{23}	E_{24}
Maltose (% w/v)	2.5	1.5	1.5	3.5	3.5	2.5	3.5	1.5	3.5	1.5
Soybean meal (% w/v)	4	2	6	2	6	4	6	2	2	6
K_2HPO_4 (% w/v)	0.6	0.2	1.0	1.0	0.2	0.6	1.0	1.0	0.2	0.2
SRP (EU/ml), Cycle 1	5,508	3,976	5,648	4,532	6,576	5,324	6,964	5,052	5,576	6,612
SRP (EU/ml), Cycle 2	5,384	3,788	5,392	4,488	6,960	5,628	7,104	4,948	5,548	6,304
SRP (EU/ml), difference	124	188	256	44	−384	−304	−140	104	28	308
SRP (EU/ml), average	5,446	3,882	5,520	4,510	6,768	5,476	7,034	5,000	5,562	6,308
Set II										
Exp no.	E_{10}	E_{11}	E_{12}	E_{13}	E_{14}	E_{20}	E_{21}	E_{22}	E_{23}	E_{24}
Maltose (% w/v)	3.5	2.5	2.5	4.5	4.5	3.5	4.5	2.5	4.5	2.5
Soybean meal (% w/v)	6	5.5	6.5	5.5	6.5	6	6.5	5.5	5.5	6.5
K_2HPO_4 (% w/v)	1.0	0.8	1.2	1.2	0.8	1.0	1.2	1.2	0.8	0.8
SRP (EU/ml), Cycle 1	6,965	5,915	6,135	7,215	7,240	6,945	5,890	6,150	7,060	6,725
SRP (EU/ml), Cycle 2	6,805	5,490	5,855	7,140	7,425	7,085	6,335	6,220	6,700	6,205
SRP (EU/ml), difference	160	425	280	75	−185	−140	−445	−70	360	520
SRP (EU/ml), average	6,885	5,703	6,721	6,821	7,333	7,015	5,890	6,784	6,680	6,792

proved to be the most suitable organic nitrogen source and an excellent inducer for SRP production (Fig. 3).

Optimization of Production of SRP Using EVOP

In designing the experiments of Set I (Table 5), the initial concentration of three variables representing the control region (E_{10}, E_{20}) was selected to be the optimum concentration of individual factors as determined from an earlier investigation. The experimental conditions for the Set I experiments, the corresponding enzyme activities of cycles I and II, and their differences and average values are presented in Table 5. The error limits, effect, and change in mean effect were calculated using the formulas given in Table 2 and are shown in Table 6. From the results of Set I (Table 5), we found that experiment E_{21} gave a maximum 7,034 EU/ml enzyme activity and E_{11} gave 3,882 EU/ml activity. It can be seen from Table 6 Set I that the change in mean effect was large and positive (104.6). The effects of maltose and of soybean meal were larger whereas that of K_2HPO_4 was smaller than the error limits for effect; hence the concentration of maltose and soybean meal were increased for the next set of experiments. Set I of Table 5 had not justified the optimum search for maximum SRP production, so a new set of experiments was designed by taking E_{21} as a new search level (Table 5).

The experimental conditions and results of Set II are presented in Table 5 and the effects are shown in Table 6. In Set II, E_{14} gave the maximum (7,333 EU/ml) and E_{21} the minimum (5,890 EU/ml) enzyme activity. It may be observed from the magnitude of effects and error limits of set II (Table 6) that the change in mean effect is −287.6. The effects of maltose (181.0), soybean meal (187.0), and K_2HPO_4 (−73.0) were lower than the error limit for effect (191.2). The effect of maltose×soybean meal×K_2HPO_4 (−108.0) suggested the possibility of interaction among them. Analysis of Tables 5 and 6 showed that the optimum concentrations of maltose, soybean meal, and K_2HPO_4 were 4.5%, 6.5%, and 0.8% (w/v), respectively.

Table 6. Magnitude of effects and error limits of set I and set II.

	Set I	Set II
Effect of maltose	753.5	181.0
Effect of soybean meal	1,706.5	187.0
Effect of K_2HPO_4	−151.5	−73.0
Effect of maltose×soybean meal	158.5	−326.0
Effect of maltose×K_2HPO_4	−241.5	−578.0
Effect of soybean meal×K_2HPO_4	−184.5	−684.0
Effect of maltose×soybean meal×K_2HPO_4	843.5	−108.0
Change in mean effect	104.6	−287.6
Standard deviation (σ)	187.6	190.5
Error limits for averages	214.0	267.4
for effects	188.3	191.2
for change in mean	167.1	169.7

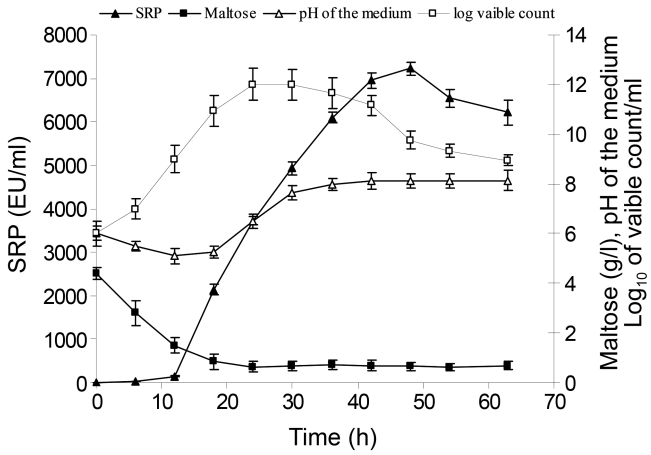

Fig. 4. Production profile of SRP with EVOP-optimized medium.

Production Profile of SRP with EVOP-Optimized Medium

Production and utilization curve were determined using EVOP-optimized media (Fig. 4). It was observed that SRP was not produced until 12 h. Maximum production was achieved in 48 h, after which a decrease in SRP titer was observed. This could be due to degradation of the enzyme at high pH. Almost 85% maltose was utilized within 20 h of fermentation. As the carbon source was utilized (0 to 20 h) by the bacterium, the pH of the fermentation broth decreased, probably owing to acid production (primary metabolite). After 20 h, there was no significant utilization of carbon source, which eventually led to a slow rise in pH, possibly due to the production of some secondary metabolites.

Discussion

To the best of our knowledge, the use of a statistical technique for production of SRP is not reported in the literature. *S. marcescens* NRRL B-23112 was used in this optimization study.

An initial pH of 6.0 of the fermentation medium supported maximum SRP production. Several workers have reported pH of 7.0−7.5 to be optimum for the production of SRP [7, 17, 21]. Our observation of pH 6.0 to be optimum can be explained as follows: from Fig 2, it is clear that as the initial pH of the fermentation medium increased from 5.5 to 8.5, the pH after fermentation was also gradually increased toward the alkaline pH of 8.1 to 9.2, respectively. It is speculated that such high alkaline pH of 8.5 to 9.2 was not favorable for the growth and production of SRP, and may have led to early onset of the stationary phase. This

evidence is also supported by the DCW and protein content, which also followed the same pattern as that of SRP.

Among all the carbon sources screened, maltose was found to support reproducible and maximum production of SRP. Moreover, none of the carbon sources exhibited substrate inhibition in the concentration range used in the study.

The organic nitrogen source has an important role in induction of SRP. Tryptone, peptone, and skim milk have been reported as good inducers of metalloprotease [5, 7, 8]. Many workers have reported SRP (metalloprotease) production to be dependent on whey protein. According to these workers, SRP is produced as the major proteases and serine protease as the minor protease, only in the presence of whey protein as a media component and *vice versa* if whey protein was substituted with any other organic nitrogen sources [19, 20, 23]. Our results showed no such phenomenon, and SRP was in fact induced by most organic nitrogen sources used in the study. Further to the production of serine protease with different organic nitrogen sources, the inhibition of 92–98% protease activity with 5 mM EDTA indicated the presence of a high level of SRP (metalloprotease) in the fermentation broth, and there was no other protease contaminant produced along with SRP in the fermentation medium.

A powerful statistical design, EVOP, was applied to optimize the significant medium component for SRP production. From the analysis of the results given in Table 6 Set I, it can be seen that the change in mean effect is large and positive (104.6); a large and negative change in mean effect as compared with error limits is desirable for determination of maximum enzyme activity. However, as per the decision-making rule, the interaction effects should also be less than the error limit, which was not fulfilled [2, 3]. Therefore, the conditions at the search region (E_{10}, E_{20}) in Set I indicated that the optimum had not been reached. It was then necessary to select a new search region to design the next set (Set II) of experiments. One simple approach of doing so was to choose the best experimental condition giving the maximum enzyme activity from the completed set of experiments (here Set I) as the search level for experiments (E_{10}, E_{20}) of Set II. A more logical approach would be to first examine the magnitudes of the individual effects of the interactions and then decide on increasing or decreasing the concentration of the individual factors. From the results of Set I, we found that experiment E_{21} gave the maximum enzyme activity. Therefore, the condition at E_{21} could be chosen for the search level (E_{10}, E_{20}) for the next set of experiments (Set II). On analyzing the magnitudes of the individual effects of interactions from Table 6, the effects of maltose and soybean meal are seen to be positive and large compared with the error limit, which indicates that the increase in maltose and soybean meal concentrations in the fermentation medium may be the desired requirement. Similarly, the effect of K_2HPO_4 is negative and within the range, as compared with the error limit, and hence found to be the optimum level for K_2HPO_4. However, on comparing the levels of E_{10} and E_{21} of Set I, the concentration of K_2HPO_4 in E_{21} is higher [1% (w/v)] than in E_{10} [0.6% (w/v)]. An increase of K_2HPO_4 in the fermentation medium may improve SRP activity. From the above analysis, it can be said that the selection of experimental conditions at E_{21} of Set I as the search level of Set II is justified. It may be observed from the magnitude of effects and error limits of Set II (Table 5) that the change in mean effect is negative and large compared with the error limit, and this satisfies one basic requirement for finding the optimum concentrations of the variables for enhancing production of SRP. The effects of maltose, soybean meal, and K_2HPO_4 are smaller than the error limit, which indicates that further change in its concentration would not be desired. The combined effect of factors in Set II was also substantially low compared with the error limit [*viz*., effect of K_2HPO_4, effect of maltose×soybean meal×K_2HPO_4 (–108.0)], suggesting the possibility of interaction among them.

The present study shows that the judicious and effective evolutionary optimization of fermentation parameters of serratiopeptidase led to serratiopeptidase titers that is, to the best of our knowledge, amongst the highest recorded to date in the scientific literature, on a shake-flask level. Moreover, the SRP production was not found to be dependent on whey protein and was free from other contaminant proteases, unlike that reported in the literature. Further scale-up studies on a bioreactor level for enhancing serratiopeptidase production are in progress.

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