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Studies on Improving the Immobilized Bead Reusability and Alkaline Protease Production by Isolated Immobilized *Bacillus circulans* (MTCC 6811) Using Overall Evaluation Criteria

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Abstract This study uses an overall evaluation criterion for improving the immobilized bead reusability and extracellular enzyme production by immobilized cells by assigning relative weightage to bead reusability, enzyme production, and cell leakage. Initially, alkaline protease production by alginate-immobilized *Bacillus circulans* (MTCC 6811) was analyzed using L18 orthogonal array (OA). The resultant optimized parameters were further fine-tuned with L9 OA experimentation. At L18-OA analysis, inoculum level and $CaCl_2$ had least influence at individual level. At the interactive level, incubation time revealed maximum and minimum interaction with sodium alginate and glucose concentration, respectively. L9 experimentation indicated that glucose concentration contributed the major influence on protease production followed by matrix material and incubation time at the individual level, and at the interactive level, matrix concentration played a vital role by interacting with incubation time, inoculum, and $CaCl_2$ concentration. All selected input parameters showed significance either at individual level or interactive in both OAs. Scanning electron microscopy analysis showed bacterial morphology variation with variation of matrix concentration. Overall, glucose concentration depicted a major influence at the individual level for the enzyme production**.** Significant improvement, approximately 147%, in enzyme yield was observed. Economic enzyme production by immobilized *B. circulans* is regulated by interactive influence of fermentation parameters, which influence the immobilized bead stability, reusability, and enzyme yield.

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Introduction

In the present biotechnological era, microbial research is playing a vital role in industrial development. Alkaline proteases are robust enzymes with considerable biotechnological potential for industrial sectors like detergents, processing of leather, recovery of silver, medical purposes, food processing, feeds and chemical industry, as well as in waste treatment [1–3]. The global proteolytic enzyme demand should reach to 22.0 billon dollars by 2009 [4]. Although these enzymes have been extensively investigated since the emergence of enzymology, there is a renewed interest because of their catalytic functional recognition especially for cellular metabolic processes as signaling molecules of numerous vital processes, as well as in pharma and other sectors for the development of high value-added product production by protease-aided digestion [4–6]. This emphasizes isolation of new microbial strains for novel proteases, characterization, and development of low-cost industrial media formulations for enzyme production [7, 8]. In this respect, an efficient alkaline protease-producing bacterial strain was isolated, characterized, and identified as *Bacillus circulans* in our laboratory [7]. The same strain was deposited in microbial type culture collection, Chandigarh, with an accession no. MTCC 6811. Preliminary experimental data based on one-factor-at-a-time approach revealed that alkaline protease production by this strain is influenced by incubation temperature and time, pH of the medium, inoculum level, and carbon and nitrogen source concentration [9]. Our laboratory data also revealed that alkaline protease produced by this strain showed effective dehairing property (unpublished data).

To sustain protease production at an industrial level, as productivity is the one of the major objectives for potential microbial strain, requires understanding the basic needs of the microbial strain to achieve a maximum output [8]. Several different approaches such as cellular metabolic regulation, use of different fermentation strategies, and selection of suitable nutrient components and fermentation parameters have been observed as components to enhance the productivity with minimum nutrient input for several microbial strains [8, 10–13]. Whole-cell immobilization appears to be an interesting alternative to conventional free-cell fermentation because of easier downstream processing without loss of biomass, increased productivity, and operational stability or reducing the fermentation time in enzyme production [12, 14–16]. Here too, fermentation process parameters optimization, using software-mediated optimization methodologies play an important role in any technological development for economic productivity in the present competitive biotechnological era. Literature reports denoted that optimization of process-related parameters (fermentation conditions, nutrient components, and biosystem-mediated) led to enhance the productivity with free-cell fermentations [8, 13, 17–19]. However, fermentations with immobilized cells need extra attention, as bead stability and productivity depend on nutrient components-mediated cellular metabolism and subsequent biomass, as well as product production in addition to other bioreactor operational parameters [12, 20]. Reusability of immobilized beads is one of the major parameters for improving the economics of the process. Reusability depends on the sustained integrity of the bead, which is influenced by microbial cell growth inside the bead, other nutrient components interactive nature with the immobilization matrix, and mass transfer [10, 12]. Conventional optimization procedures are time consuming and cannot provide information on mutual

interactions of the parameters. Statistical optimization procedures have advantages over conventional methodologies by achieving optimization more quickly and are able to evaluate interactions. Taguchi methodologies are developed based on orthogonal arrays (OAs) and can provide three phases of offline quality controls (i.e., system, parameter, and tolerance design). System design helps to identify the working levels of design factors. While parameter design indicates the factor levels that give the best performance of the product/process under study, tolerance design helps to fine-tuning factors that significantly influence the quality of product formation. This Taguchi method has advantage in considerable saving in time and cost because it does not use a full factorial analysis. Taguchi methodologies use several design arrays such as OA12, OA 18, OA 36, and OA 54, which enable a focus on the main effects and help in increasing the efficiency of the overall process [13].

To enhance enzyme production with the immobilized *B. circulans* strain, an overall evaluation criteria (OEC) as defined by the Taguchi methodology was used to optimize three major objectives, namely reduction of biomass production in the medium during immobilized cell fermentation, enhancement of enzyme productivity, and increase of immobilized cell bead reusability.

Materials and Methods

Microorganism and Growth Conditions

A bacterial strain used in this investigation, was isolated from the soil samples collected from Vishakapatnum, Andhra Pradesh, India, using 2% agar-based 1% casein containing medium by serial dilution method. The inoculated agar plates were incubated at 33 °C for 24 h in an incubator. Based on casein hydrolysate zone, several colonies were selected and studied in detail. One of the colonies, showing maximum zone formation was further characterized for growth and protease production values. Microbial Type Culture Collection (MTCC), Chandigarh, identified the selected strain as *B. circulans*. Subsequently the strain was deposited in MTCC and provided an accession no. MTCC 6811. The bacterial strain was grown using yeast extract–peptone–glucose (YPG) medium (pH 9.0) containing glucose—10.00, peptone—7.5, yeast extract—7.5 (in g/l), and 5.0 ml of salt solution [salt solution prepared by adding (in grams) $MgSO_4.7H_2O$—5.0, KH_2PO_4—5.0, and $Fe(SO_4)_2.7H_2O$—0.1 in 1 l of distilled water] by incubating at 33 °C for 24 h in an orbital shaker (LabTech—LSI-3016 R) adjusted to 150 rpm. All experiments were carried out in 250-ml Erlenmeyer flasks containing 50-ml fermentation medium. The organism was maintained by subculturing at regular intervals on agar-based YPG medium slants and stored at 4 °C until further use.

Preparation of Immobilized Cells

A medium viscosity (500 mPa s) sodium alginate was purchased from Sigma Aldrich Ltd. and used in this study. All the glassware and solutions used in the protocols were sterilized at 121 °C for 20 min. Immobilized cell beads were prepared according to the method outlined in Bashan (1986) [21] with modifications under sterile conditions. Predetermined quantities of sodium alginate (1.75, 2.0, 2.25, 2.5, 2.75 g) was dissolved in 80 ml of distilled water separately and sterilized. Log phase (18 h grown) *B. circulans* cell mass was harvested by centrifugation (6,000×*g*, 10 min) and washed twice with sterilized saline

(0.9% NaCl, *w*/*v*) solution. *B. circulans* cell pellet was diluted to achieve a cell density of 5.0×10^8CFU/ml using sterilized distilled water. Twenty milliliters of this cell suspension was added to each sodium alginate solution and mixed thoroughly. This suspension was extruded dropwise through a 10-ml syringe with a 19-gauge needle into a precooled sterile 2.0%, 3.0%, and 4.0% (*w*/v) aqueous solution of $CaCl_2$ under mild agitation as per the experimental design to get water-insoluble calcium alginate beads with bacterial cells. These beads were incubated for 3–6 h at room temperature to achieve effective polymerization and washed twice with sterile distilled water before being used as inoculum for protease production. Approximately 220 beads/50 ml medium, unless otherwise stated, were used for each experiment.

Measurement of Alkaline Protease Activity

Alkaline protease activity in cell-free fermentation broth (as enzyme source) was determined using modified Auson–Hagihara [22] methodology. The enzyme reaction was initiated by adding 0.1 ml of enzyme source to the reaction mixture consisting of 1.0 ml of 1% (*w*/*v*) casein dissolved in glycine–NaOH buffer (50 mM, pH 11.0) and 0.9 ml of 70 °C preincubated glycine–NaOH buffer (50 mM, pH 11.0) and incubated at 70 °C. After 20 min of incubation, adding 2 ml of 10% trichloroacetic acid the enzyme activity was terminated. The precipitated unhydrolyzed casein was removed by filtration using Whatman filter paper no. 1, and absorbance of the filtrate was measured at 280 nm using UV-visible spectrophotometer (Perkin-Elmer λ25). The protease activity was measured in terms of tyrosine released using a tyrosine standard curve. One unit of the alkaline protease activity was defined as the amount of enzyme required in liberating 1 μg of tyrosine ml^{-1} under experimental conditions. All experiments were conducted in triplicates, and results reported here were average values having 3% experimental error.

Enumeration of *B. circulans* Cells

To estimate the viable counts, the encapsulated bacteria were released from the beads by dissolving five beads in phosphate buffer (pH 7.0) for 30 min followed by gentle homogenization. The bacterial count in this solution was measured after serial dilution and by plating agar-based fermentation medium using standard plate count method after incubating at 33 °C for 24 h.

Scanning Electron Microscopy

For microscopic studies, immobilized beads containing *B. circulans* cells were transferred to vials and fixed using 3.5% glutaraldehyde solution for 2 h at 4 °C. The samples were then dehydrated using an alcohol gradient series. The air-dried samples were then mounted over the stubs with double-sided adhesive tape. Finally, a thin layer of gold was coated over the sample using HUS-5GB Hitachi vacuum evaporator for about 90 s. The samples were then scanned under scanning electron microscope (SEM), Hitachi, S-3000N, at various magnifications at an acceleration voltage of 10.0 kV.

Software Package

Qualitek-4 software (Nutek Inc., MI) for automatic design of experiments using the OEC approach of the Taguchi methodology was used in the present study. Qualitek-4 software is

equipped to use L-4 to L-64 arrays along with the selection of two to 63 factors with two, three, and four levels to each factor, mainly to reduce experimental errors and to enhance the efficiency and reproducibility of the laboratory experiments. The automatic design option allows Qualitek-4 to select the particular array and assign factors to the appropriate columns. This software was used to study (a) the identification of the individual influence of each factor, (b) OEC, (c) determination of the optimum condition, and (d) estimation of performance at the optimum condition.

OEC Methodology (OEC_M)

OEC methodology adopted in this study was selected from Qualitec-4 software that was divided into various steps, viz., planning, experimentation, analysis, and validation, with defined objective, and interconnected in sequence to achieve the overall optimization process. The schematic representation of the designed methodology is depicted in Fig. 1.

Design of Experiments

The first step in phase 1 is to determine the various factors to be optimized in the culture medium that have a critical effect on the protease production in conventional methodology. The normal practice is to experiment with the feasible range, so that the variation inherent in the process does not mask the factor effect. Based on detailed fermentation experiments with immobilized *B. circulans*, cell beads were carried out using the one-factor-at-time approach experimental data, five factors (inoculum level, incubation time, sodium alginate, $CaCl_2$, and glucose concentrations) having significant influence on the immobilized bead stability, and protease production were selected along with ranges for the present Taguchi design of experiments study. The next step was to design the matrix experiment and to define the data analysis procedure. The appropriate OAs for the control parameters to fit a specific study was selected. In the present study, three levels of factor variation were considered, and the size of experimentation was represented by symbolic array L-18 (which indicates 18 experimental trails). Five factors with three levels were used, and these are depicted in Tables 2 and 3. The total degrees of freedom is equal to the number of trails minus 1, i.e., 17. In the design OA, each column present a number of conditions depending on the levels assigned to each factor. Based on the optimal conditions and performance-predicted conditions, further optimization experiments were carried out using factors that showed extreme selected levels as optimum concentration. Incubation time and glucose and sodium alginate concentration were evaluated for three different levels using L9 OA.

Fermentation Experiments with Immobilized Cell Beads

Fermentation experiments were performed using sodium-alginate-immobilized *B. circulans* cell beads, employing the selected L18 (Table 3) and L9 (Table 9) experimental trails in combination with selected factors at three levels (Table 2 and 8). The protease activities and cell number in the medium presented in the table are the average of the three determinations.

Analysis of Experimental Data and Prediction of Performance

Results were analyzed for three objectives (protease production, immobilized bead reusability, and cell number in medium) using the OEC technique from the Taguchi

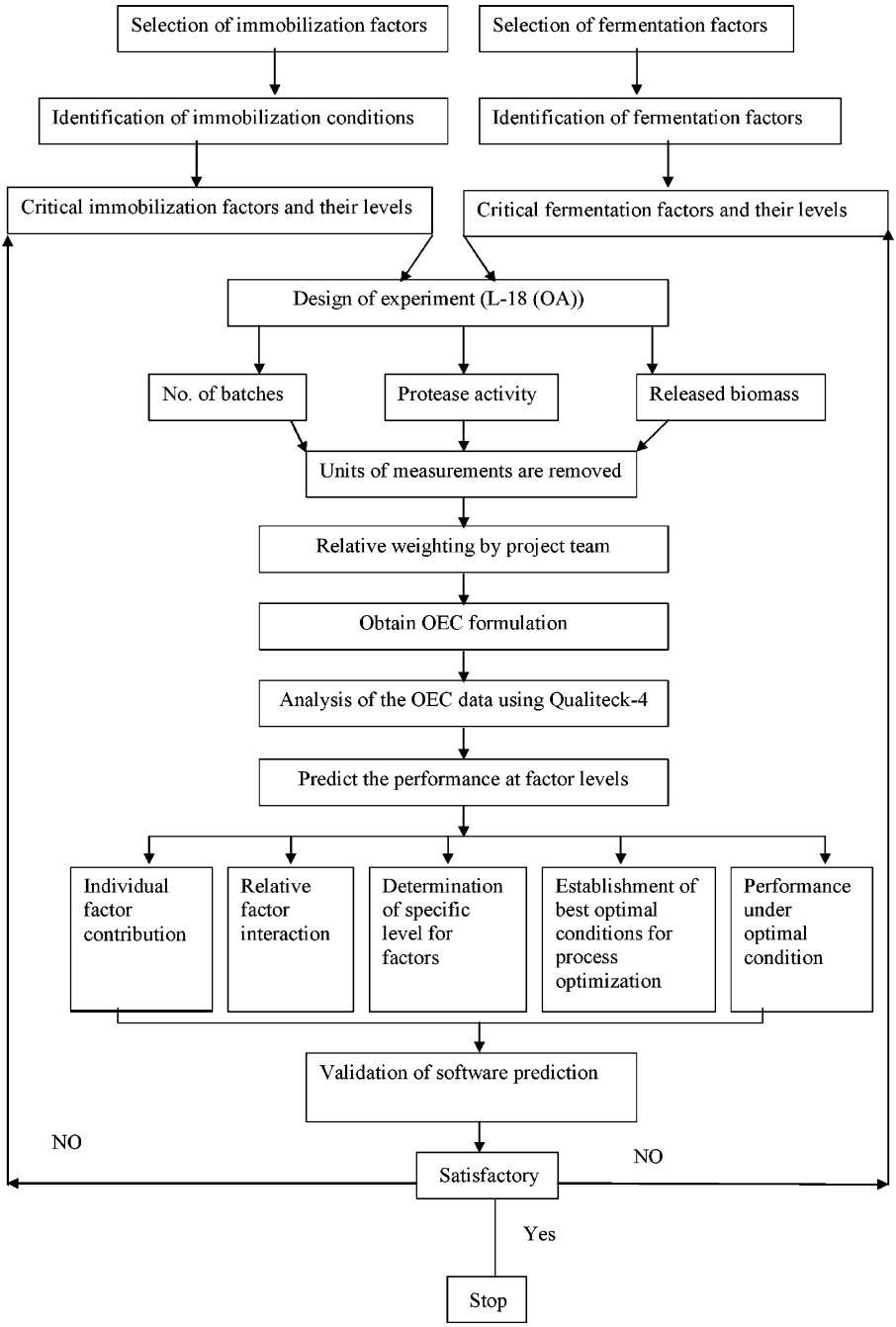

Fig. 1 Schematic representation of experimental step up

methodology using the Qualitek-4 software. These objectives have different quality characteristics (QC), with bigger-is-better quality characteristic for protease production and immobilized bead reusability and smaller-is-better for cell number in medium. Because these QC are important for improving the economy of the process and demand proper consideration to meaningful combination of evaluation from different criteria, these QC are transformed into a single index to optimize the enzyme yield using OEC technique [23, 24] based on Eq. 1 to identify the influence of individual factors and to estimate the performance at the optimum conditions (Table 4) in following steps.

$$\left(\mathrm{OEC}\right)11 = \left(\frac{|\mathrm{S1} - \mathrm{W1}|}{|\mathrm{B1} - \mathrm{W1}|}\right) \times \mathrm{RW1} + \left(\frac{|\mathrm{S2} - \mathrm{W2}|}{|\mathrm{B2} - \mathrm{W2}|}\right) \times \mathrm{RW2} + \left(1 - \frac{|\mathrm{S3} - \mathrm{W3}|}{|\mathrm{W3} - \mathrm{B3}|}\right) \times \mathrm{RW3} \tag{1}$$

where W = worst value, B = best value, RW = relative weightage, S = sample value, 1 = reusability, 2 = protease activity, and 3 = bacterial cells released in fermentation medium with respect to W, B, RW, and S.

In step 1, the results (enzyme in *units* and immobilization bead reusability in *number of cycles*, and cells in the fermentation medium in *numbers*) from individual evaluations were normalized, i.e., freed from their units (dimensionless). In step 2, contributions from all readings were measured to the QC, either bigger or smaller (in this study, bigger QC is better for enzyme production and immobilization cycles, and smaller is better for cell leakage). In step 3, individual readings are converted in proportion to the relative weightage (degree of importance) as determined subjectively by assigning higher relative weighting to enzyme production, immobilization cycles, and less relative weighting to cells in the fermentation medium. All these steps are used in OEC using Qualitek-4 software for enzyme production process evaluation. In Taguchi's method, quality is measured by the deviation of a characteristic from its target value as a loss function [$L(y)$] and was represented by

$$L(y) = k(y - m)2\,, \tag{2}$$

where k denotes the proportionality constant, m represents the target value, and y is the experimental value obtained for each trail. In the case of bigger-is-better-quality characteristics, the loss function can be written as

$$L(y) = k(1/y2) \tag{3}$$

and the expected loss function can be represented by the results obtained from the data processing.

Validation

To validate the methodology, fermentation experiments were performed in triplicate using the software-predicted optimized culture conditions as identified in Tables 7 and 12 for L18 and L9 OA experiments, respectively.

Results

Alginate concentration showed influence on repeated use of immobilized bead (stability) and on cell mass in the medium during fermentation (Table 1). Better protease production

Table 1 Effect of immobilization matrix concentration on the bead stability and alkaline protease production by immobilized *B. circulans*.

S. no	SA (%, *w*/*v*)	Protease activity (U/ml)		Cell number in medium		Cells with in the beads		No. of batches	Productivity (U/ml per h)	
		Mean ± SE	*p* Value	Mean ± SE	*p* Value	Mean ± SE	*p* Value		Mean ± SE	*p* Value
1	1.5	5,150±26	0.000026	$6.34\times10^{8}\pm1.5\times10^{6}$	0.000600	$2.2\times10^{6}\pm 5.7\times10^{4}$	0.000682	6	214.58±1.10	0.000026
2	2.0	4,900±21	0.000020	$4.91\times10^{5}\pm1.1\times10^{4}$	0.000543	$6.4\times10^{6}\pm 1.4\times10^{5}$	0.000501	10	204.16±0.90	0.000020
3	2.5	4,450±19	0.000018	$3.36\times10^{5}\pm7.4\times10^{3}$	0.000489	$3.87\times10^{5}\pm6.7\times10^{3}$	0.000303	11	185.40±0.79	0.000018

SE standard error, *SA* sodium alginate

and large number of bacterial cells were noticed with immobilized cell beads prepared with 1.5% alginate than with the other two concentrations. Increase in alginate concentration from 1.5% to 2.0% improved the reusability of beads from six to ten cycles without much variation in enzyme production. Slight improvement (10%) in reusability of immobilized beads with 10% reduction in enzyme production was observed with an increase of alginate concentration to 2.5%. This data suggested the matrix concentration importance in bead stability or reusability.

Bacterial cell population in the fermentation medium and protease production was decreased with increase in alginate concentration in immobilized cell beads. The maximum cell mass (number of cells) was found in immobilized beads prepared with 2.0% alginate concentration. A progressive improvement in stability of immobilized beads (from six batches to ten) was noticed with increasing alginate concentration from 1.5% to 2.0%. However, further increase in alginate concentration to 2.5% neither improved cell mass inside the bead nor protease production.

The structural morphology of alginate immobilized *B. circulans* beads was studied under a SEM. The surface texture varied from smooth (Fig. 2a) to rough with ridges and furrows along with undulated scaly microtexture as the matrix concentration increased from 1.5% to 2.0% (Fig. 2e). Similar results were observed by Beshay [25] working on alkaline protease with immobilized *Teredinobacter turnirae* and Nava et al. [26] working on the impact of immobilization conditions on gel formation, who noticed the similar type of results. Further increase in alginate concentration did not alter the surface texture (Fig. 2i). Beads containing 1.5% alginate showed solitary bacterial cells, and their distribution was uniform on the surface, as well as in the interior of the bead; however, the cell density was high in the interior than on the surface (Fig. 2a and b). Cells appeared as rods and vesicles with uniform distribution on the surface, as well as in the interior of the bead, and are firmly immobilized between the scaly structures in the beads prepared with 2.0% alginate (Fig. 2e and f). When alginate concentration increased to 2.5%, a few cells are exposed on the surface, and most of the cells are embedded by the alginate (Fig. 2i and j). After fermentation, beads produced from the 1.5% alginate indicated reduced cell density to a great extent on the surface associated with pores on the surface, as well as in the interior (indicated by arrows), and cells appeared in clusters (Fig. 2c and d). While solitary cells at the interior and dense clusters on the surface was observed with the 2.0% alginate beads after fermentation (Fig. 2g and h). Whereas 2.5% alginate-fermented immobilized beads showed enriched smaller cells on the surface and solitary vesicular cells in the interior (Fig. 2k and l).

The impact of immobilization-matrix concentration on microbial cell retainability was investigated. It was observed that the cell-retaining capacity of the immobilized bead also followed the similar trend of stability of the bead with respect to alginate concentration. Two percent alginate beads showed maximum cell retaining capacity. Variation of immobilization matrix concentration to either side of this concentration caused reduction in the number of immobilized cells. The reduction of the number of microbial cells inside the bead was more pronounced with increase of alginate concentration in the bead. Lowering of alginate concentration (0.5%) resulted in threefold reduction in the number of cells inside the beads. This reduction of cell retention with respect to alginate concentration may be attributed to the larger pore size, which causes the release of microbial cells from the bead to the fermentation medium during incubation at constant shaking environment. This was further supported by the threefold-higher number of cells in the medium with 1.5% immobilized bead fermentation than 2.0%. An increase of immobilization-matrix concentration from 2.0% to 2.5% also resulted in the reduction of cell retention in the

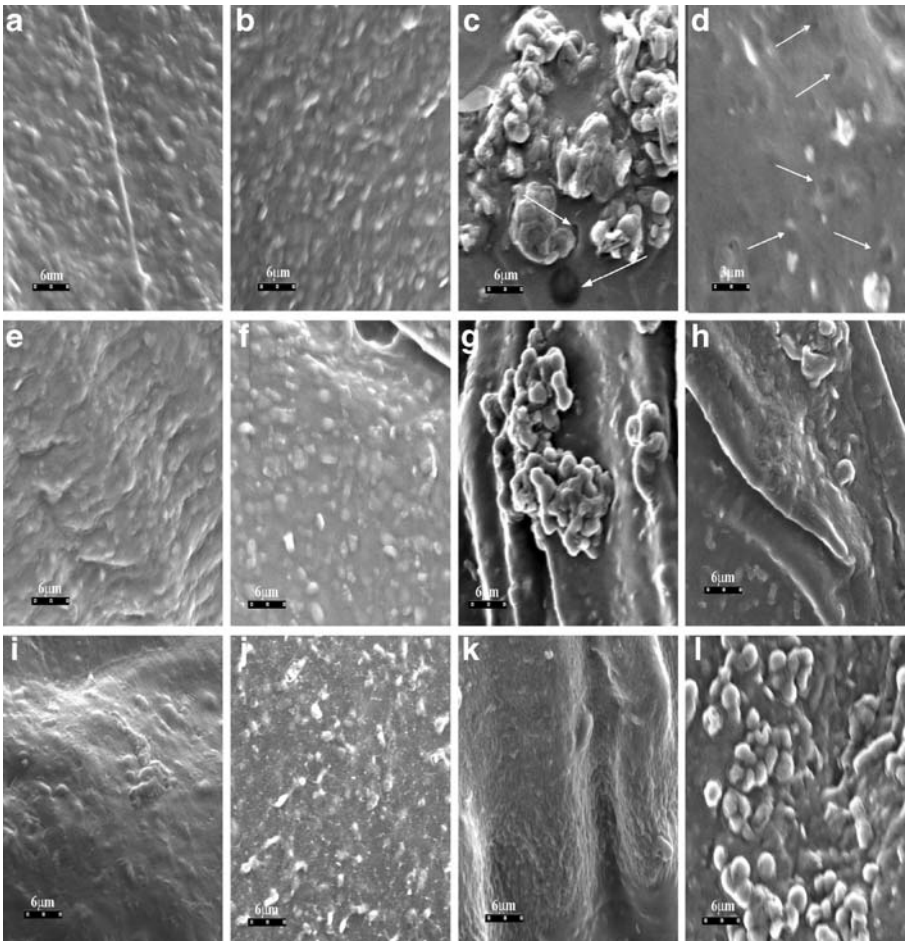

Fig. 2 Scanning electron micrograph pictures of immobilized beads before and after fermentation **a**–**d** for 1.5%, **e**–**h** for 2.0%, and **i**–**l** for 2.5%. **a**, **e**, and **i**, and **c**, **g**, and **k** Surface pictures before and after fermentation, respectively. **b**, **f**, and **j**, and **d**, **h**, and **l** Cross-section pictures before and after fermentation, respectively

immobilized bead. The total cell number present in the 2.5% alginate bead was $3.87\times10^5\pm 6.7\times10^3$ per bead, while 1.5% alginate bead retained only $2.21\times10^6\pm5.7\times10^4$ cells per bead with a statistical significant difference of $p=0.000303$ and 0.000682, respectively. However, decrease in cell numbers in 2.5% alginate beads compared to 2.0% may be attributed to the change of structural integrity of the immobilized bead at this matrix concentration. This may be attributed to the fact that as the alginate concentration varies, the density of the material also varies, resulting to lower porosity at higher matrix concentration. Repeated fermentations did not alter the number of cells present in the immobilized bead up to 11 fermentation cycles. The variation observed in cell number in the bead was in the range of ±5%. Further use of these immobilized beads resulted in gradual reduction in the number of cell per bead associated with progressive reduction of protease production, and this was continued till the bead was dissolved (results not shown). The reduced enzyme production by immobilized beads prepared using lower concentration

of alginate may be attributed to lower cell retainability, as it could be visualized based on the observation that very limited number of cells present after fermentation with the beads prepared from the 1.5% alginate (Fig. 2d) compared to those from the 2.5% (Fig. 2l). From this data, it was clear that protease production with 2.0% alginate immobilized *B. circulans* strain is economical especially without loss of productivity.

A further study was performed to achieve maximum alkaline protease productivity by optimizing multilevel objectives. During this study, two different process parameters, i.e., immobilization process factors (matrix and poly-electrolyte concentrations) and fermentation process parameters (glucose concentration, inoculum level, and incubation time), were considered for achieving the optimum alkaline protease productivity. Optimum concentrations where maximum alkaline protease production was noticed, were considered (based on Table 1) as middle levels (level 2 concentration), and the negative and positive sides of level 2 was considered as lower levels (level 1) and higher level (level 3 concentration), respectively, for each factor studied. Table 2 represents the selected factors and their concentrations (as levels) used for optimization of protease enzyme production by immobilized *B. circulans* cells in the selective fermentation medium.

Table 3 indicates the experimental setup and the impact of different selected immobilization and fermentation factors, which include cell mass in the medium during fermentation, bead performance or reusability, and alkaline protease production by alginate-immobilized *B. circulans* cells. Enzyme production, cell release and recycling nature of beads were varied from 5,700 and 4,200 U/ml, 1.08 to 9.52×10^5 cells, and nine to 26 cycles, respectively, depending on experimental conditions. At maximum enzyme production environment, immobilized cell beads could be used only nine cycles against a maximum of 26 cycles. Similarly, where cell leakage was at a minimum, the alkaline protease production value was reduced to 4,300 U/ml. The data was analyzed for optimization of the above three parameters toward overall improvement in enzyme productivity using OEC. Each of these parameters was initially analyzed for its minimum (worst) and maximum (best) performance based on their contribution for improving overall productivity, and the relative weightage (in percent) assigned for each objective (here reusability, number of cells in the medium, and enzyme activity; Table 4) and the data given in Table 3 were analyzed. OEC analysis identified that maximum and minimum output of 89.63 and 15.08 for the three respective experimental dimensionless values (Table 3).

The main effects of factors at the assigned levels on overall protease production were shown in (Fig. 3a–e). The difference between the average value of each factor at levels 2 and 1 indicates the relative influence of the effect. An overall improvement in economic enzyme production was noticed with increase in alginate concentration from first (1.5%) to third level (2.25%; Fig. 3a). Effective difference in overall performance of immobilized bead-associated enzyme production was observed with a variation in glucose concentration from 0.5% to 0.25% (*w*/*v*), while glucose concentration increase from 0.5% to 0.75% (*w*/*v*) did not alter enzyme production (Fig. 3c), suggesting carbon source influence on immobilized *B. circulans* metabolism-linked protease production. Lower fermentation time showed better performance (Fig. 3b), while increase in biomass (in terms of number beads; Fig. 3d) and calcium chloride concentration (Fig. 3e) from levels 2 to 3 denoted decreased performances.

The percent column of the analysis of variance (ANOVA) indicates the influence of each factor at individual level on *B. circulans* metabolism relating to protease production (Table 5). Glucose was the most significant factor (>90%) among selected factors for protease production by *B. circulans*; the rest altogether showed <10% significance at individual level. Sodium alginate had a 4.5% effect at individual level (Table 5) and showed

Table 2 Selected factors and their levels for alkaline protease production using L18 orthogonal array by immobilized *B. circulans.*

S. no	Column no.	Factor	Level 1	Level 2	Level 3
1	2	SA (%, *w*/*v*)	1.75	2.00	2.25
2	3	Incubation (h)	18	24	36
3	4	Glucose (%, *w*/*v*)	0.75	0.50	0.25
4	5	No. of beads	130	180	230
5	6	$CaCl_2$ (%, *w*/*v*)	2	3	4

the highest severity index value (61.60%) at the interactive level with incubation time (Table 6). Similarly, strong influential factor, glucose, with (>90%) at individual level depicted the least severity index percentage (3.32%) with inoculum (weaker influential factor at individual level) and incubation time (2.21%). These results suggest that the influence of one factor on protease production was dependent on the condition of the other factors, and all selected factors were essential either at individual or interactive level. Similar results were noticed in microbial related products [13, 18, 19].

Software-predicted optimum conditions and factor performance in terms of contribution for achieving maximum yield at selected levels were depicted in Table 8. Glucose concentration had the more significant role in alkaline protease production than other selected parameters. The overall predicted result at the optimum conditions was 86.257, with total contributions from all factors being 37.862, and the grand average performance was 48.394.

Further analysis revealed that inoculum and $CaCl_2$ at middle level, sodium alginate and glucose concentration at level three, and incubation time at the initial level contributed to

Table 3 L18 orthogonal array experimental plan layout and alkaline protease production, number of cells in the medium, and reusability of immobilized *B. circulans* beads along with OEC relative weightage.

Experiment no.	Column					No. of batches	Protease activity (U/ml)	Cells in medium during fermentation	Predicted	Experimental
	2	3	4	5	6				Values (relative weightage)	
1	1	1	1	1	1	14	4,700	3.25×10^5	38.90	38.60
2	1	2	2	2	2	12	5,400	7.60×10^5	40.51	44.03
3	1	3	3	3	3	25	4,200	3.76×10^5	66.10	58.58
4	2	1	1	2	2	09	5,400	9.52×10^5	41.43	32.96
5	2	2	2	3	3	09	4,900	6.91×10^5	25.52	24.26
6	2	3	3	1	1	25	4,400	18.3×10^4	62.98	65.58
7	3	1	2	1	3	17	5,200	19.6×10^4	40.01	60.72
8	3	2	3	2	1	25	5,400	13.9×10^4	78.54	89.63
9	3	3	1	3	2	19	5,000	2.79×10^5	40.23	60.91
10	1	1	3	3	2	22	5,500	2.66×10^5	77.26	81.65
11	1	2	1	1	3	13	4,300	2.22×10^5	30.32	27.47
12	1	3	2	2	1	09	5,700	8.74×10^5	38.01	40.92
13	2	1	2	3	1	09	5,150	5.18×10^5	34.15	32.20
14	2	2	3	1	2	26	4,400	11.8×10^4	65.48	69.29
15	2	3	1	2	3	10	5,200	5.78×10^5	30.36	35.60
16	3	1	3	2	3	26	4,300	10.8×10^4	81.34	67.05
17	3	2	1	3	1	09	5,000	8.93×10^5	38.48	24.22
18	3	3	2	1	2	09	4,400	4.73×10^5	38.96	15.08

Table 4 Selected objective and assigned relative weightage.

S. no	Objective/criteria description	Worst value (*W*)		Best value (B)		Selected quality criteria	Relative Weightage (RW, in %)
		L18	L9	L18	L9		
1	Reusability	9	20	26	26	B	50
2	Protease activity	4,000	4,000	5,700	5,700	B	40
3	Bacterial cells in fermentation medium	9.52×10^5	9.0×10^5	1.084×10^5	3.0×10^5	S	10

B big, *S* small

the optimum yield (Table 7). This indicates further possibilities for improvement of protease production using this bacterial strain. This was further confirmed by (a) a reduction of glucose concentration from 0.5% to 0.25% in the medium that improved the protease production (~50%; Fig. 3c) and (b) an increase of immobilization matrix concentration

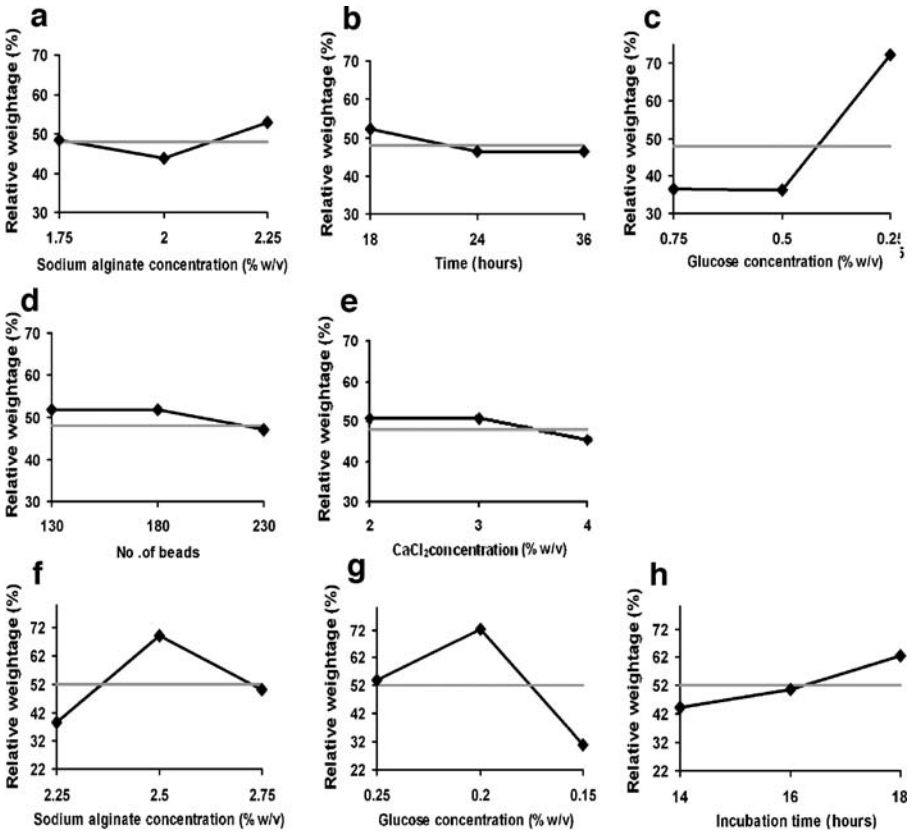

Fig. 3 Impact of selected factor assigned levels on alkaline protease production by immobilized *B. circulans* cells. *X-axis* assigned levels of selected factor, *Y-axis* relative weightage. **a** Sodium alginate, **b** incubation time, **c** glucose concentration, **d** inoculum (number of beads) and **e** $CaCl_2$ concentration for L18 OA. **f** Sodium alginate, **g** glucose concentration, and **h** incubation time for L9 OA. *Gray line* indicates the average relative weightage during experimentation and *black line* indicates the individual factor contribution on relative weightage

Table 5 ANOVA for selected fermentation factors for alkaline protease production using L18 orthogonal array by immobilized *B. circulans*.

S. no.	Factor	DOF (*df*)	Sum of squares (*S*)	Variance (*V*)	*F* ratio (*F*)	Pure sum (S)	Percent *P* (%)
1	SA	2	255.681	127.840	894,868.684	255.681	4.461
2	Incubation	2	130.111	65.055	4,553,914.206	130.111	2.270
3	Glucose	2	5,167.519	2583.759	180,863,185.396	5167.519	90.170
4	Inoculum	2	98.862	49.431	3,460,203.550	98.862	1.725
5	$CaCl_2$	2	78.657	39.328	2,753,023.558	78.657	1.372
	Other/error	7	0.001	0.001			0.002
	Total	17	5,730.834				100

DOF degrees of freedom

from 2.0% to 2.25% that enhanced the enzyme production (Fig. 3a); however, further increase to 2.5% caused reduction (Table 1), and (c) software-predicted optimum conditions revealed that the selected 18 h of incubation time was better compared to the other two incubation time (24 and 36 h) under given experimental conditions, and (d) microbial product production was reported to depend on medium-component concentration in several microbial systems [8, 10, 13,18, 19, 27]. Hence, further optimization experiments were performed using the factors that showed extreme selected levels in the above optimization studies (Table 7). L9 (OA) was used to evaluate incubation time, sodium alginate, and glucose concentration role at selected levels on improvement of protease productivity in this bacterial strain (Table 8). The experimental layout along with experimental results for reusability, number of bacterial cells in the medium during fermentation and protease activity were depicted in Table 9. Enzyme production varied from 4,000 to 5,700 U/ml, while bacterial cell population in the medium differed from 3.0×10^5 to 9.0×10^5 CFU/ml, and reusability of immobilized beads were varied from 20–26 batches depending on fermentation conditions. Comparative evaluation of experimental data revealed that

Table 6 Interactive influence between selected factors on alkaline protease production using L18 orthogonal array by immobilized *B. circulans*.

S. no	Interacting factor pairs (in order of severity)	Columns	SI (%)	Col	Opt
1	SA × incubation	2×3	61.6	1	(3, 1)
2	SA × inoculum	2×5	43.67	7	(3, 2)
3	SA × $CaCl_2$	2×6	32.42	4	(3, 3)
4	Incubation × $CaCl_2$	3×6	29.15	5	(1, 3)
5	Glucose × $CaCl_2$	4×6	22.88	2	(3, 1)
6	Incubation × inoculum	3×5	21.25	6	(2, 2)
7	Inoculum × $CaCl_2$	5×6	18.22	3	(3, 2)
8	SA × glucose	2×4	15.45	6	(3, 3)
9	Glucose × inoculum	4×5	3.32	1	(2, 3)
10	Incubation × glucose	3×4	2.21	7	(2, 3)

Columns the column locations to which the interacting factors are assigned; *SI* interaction severity index (100% for 90° angle between the lines, 0% for parallel lines); *Col* column that should be reserved if this interaction effect were to be studied (two-L factors only); *Opt* the factor levels desirable for the optimum conditions (based strictly on the first two levels). If an interaction is included in the study and found significant (in ANOVA), the indicated levels must replace the factor levels identified for the optimum condition without considerations of any interaction effects

Table 7 Optimum conditions and their performance on alkaline protease production using L18 orthogonal array by immobilized *B. circulans*.

S. no.	Factor	Level description	Level	Contribution
1	SA	4	3	04.54
2	Incubation	18	1	03.80
3	Glucose	0.25	3	23.96
4	Inoculum	1.0	2	03.30
5	$CaCl_2$	2.0	2	02.25

Total contribution from all factors=37.862, current grand average of performance=48.394, expected results at optimum conditions=86.257

variation in the number of cycle beads were reused, and cells in medium and enzyme productivity values were reduced from the L18 (Table 3) experiment than the L9 (Table 9). The overall performance of immobilized *B. circulans* in terms of enzyme production was analyzed, giving similar relative weightage to L9 experimental data, as mentioned in Table 4. A small variation was noticed between predicted and experimental relative weightages (Table 9). Further analysis at individual selected factor level indicated that better enzyme production could be possible with 2.5% immobilized beads in the presence of 0.2% glucose concentration in 18 h of fermentation (Fig. 3f–h). Based on experimental conditions, it varied from 26.66–98.91% and 24.41–96.66% for predicted and experimental weightage, respectively.

The impact of each parameter in the L9 designed experiment at individual level was analyzed using ANOVA. The data revealed that glucose had the maximum influence (56%) followed by sodium alginate (32%; Table 10) against 90% influence at L18 experimentation with five selected factors (Table 5), indicating that the effect of each factor on protease production depends on other selected factors and their concentration. Interaction analysis suggested that maximum interaction (37.04) was noticed between glucose concentration and incubation time (Table 11). This was followed by interaction between immobilization matrix material (sodium alginate) concentration and incubation time, which was 6.24%. Least interaction was noticed between glucose and sodium alginate concentrations during fermentation.

Optimum conditions and overall performance of selected parameters with respect to enzyme production are given in Table 12. Maximum productivity could be achieved using 2.5% alginate *B. circulans*-immobilized beads in fermentation medium consisting of 0.2% (*w*/*v*) glucose in 18 h of incubation. The total contribution from the above three parameters was 47%. Expected performance of optimized conditions was 98.914. Validation of these experiments was performed using three replicates and results indicated that immobilized *B. circulans* beads prepared using 2.5% sodium alginate and 2.0% $CaCl_2$ solution were stable for 26 batches with enzyme production values of 5,680±30 U/ml, indicating 315±1.25 U/ml per h productivity, suggesting >45% improvement after optimization.

Table 8 Selected factors and their levels for optimization of alkaline protease production by immobilized *B. circulans* for L9 OA experiments.

S. no.	Column no.	Factor	Level 1	Level 2	Level 3
1	1	SA	2.25	2.50	2.75
2	2	Glucose	0.25	0.20	0.15
3	3	Incubation	14	16	18

Table 9 Experimental layout and results along with predicted and experimental overall relative weightage for optimization of alkaline protease production by immobilized *B. circulans* for L9 OA experiments.

Experiment no.	Column			No of batches	Protease activity (U/ml)	Released cells	Predicted	Experimental
	1	2	3				Overall relative weightage (%)	
1	1	1	1	22	4,400	7.0×10^5	31.66	29.41
2	1	2	2	23	5,200	8.0×10^5	56.36	54.90
3	1	3	3	20	5,300	9.0×10^5	26.85	30.58
4	2	1	2	26	4,600	4.0×10^5	68.72	72.45
5	2	2	3	26	5,700	5.0×10^5	98.91	96.66
6	2	3	1	20	5,400	6.0×10^5	39.40	37.94
7	3	1	3	26	4,000	3.0×10^5	61.46	60.00
8	3	2	1	26	4,300	4.0×10^5	61.66	65.39
9	3	3	2	21	4,400	5.0×10^5	26.66	24.41

Discussion

Immobilization technology for the production of extracellular enzymes by microbes offers many advantages such as reusability of the biomass, continuous operation without cell loss, and increased productivity [16, 26]. However, proper selection of different components of immobilization, as well as the fermentation parameters, is one of the essential requirements to minimize the immobilization-associated disadvantages. Hence, in this study, the impact of sodium alginate concentration on the performance of immobilized *B. circulans* in terms of protease production, reusability of the immobilized beads, and free bacterial cell population in the medium was studied and optimized for economic production of alkaline protease by alginate immobilized *B. circulans* cell beads under submerged fermentation environment using YPG medium.

The immobilization matrix concentration affected the *B. circulans* cellular-metabolism-associated protease production and immobilized bead stability. This was evidenced based on the observation that (a) the gel network varied (Fig. 2a, e, and i) without much variations in cell distribution (Fig. 2b, f, and j) with the variation of matrix concentration before fermentation); (b) observed variations in the bead structure (appearance of holes in Fig. 2d; indicated by arrows) prepared with lower (1.5%) alginate concentration compared with higher (2.0% and 2.5%; Fig. 2d, h, and l) alginate concentration after fermentation; (c) enzyme production affects cell mass inside the bead and in the fermentation medium and bead stability (Table 1) variations associated with change in alginate concentration during

Table 10 ANOVA for selected fermentation factors for alkaline protease production by immobilized *B. circulans* for L9 OA experiments.

S. no	Factor	DOF (*df*)	Sum of squares (*S*)	Variance (*V*)	*F* ratio (*F*)	Pure sum (*S*)	Percent *P* (%)
1	SA	2	1,452.080	726.04	520,806.84	1,452.08	31.98
2	Glucose	2	2,573.199	1,286.59	731,999.84	2,573.19	56.67
3	Incubation	2	515.178	257.58	151,785.13	515.17	11.34
	Other/Error	2	.001	.001			0.002
	Total	8	4,540.459				100

Table 11 Interactive influence between selected factors on alkaline protease production by immobilized *B. circulans* for L9 OA experiments.

S. no	Interacting factor pairs (in order of severity)	Columns	SI (%)	Col	Opt
1	Glucose × incubation	2×3	37.04	1	(2, 3)
2	SA × incubation	1×3	6.24	2	(2, 3)
3	SA × glucose	1×2	0.88	3	(2, 2)

Columns the column locations to which the interacting factors are assigned; *SI* interaction severity index (100% for 90° angle between the lines, 0% for parallel lines); *Col* column that should be reserved if this interaction effect were to be studied (two-L factors only); *Opt* the factor levels desirable for the optimum conditions (based strictly on the first two levels). If an interaction is included in the study and found significant (in ANOVA), the indicated levels must replace the factor levels identified for the optimum condition without considerations of any interaction effects

immobilized *B. circulans* cell bead preparation; (d) increase in enzyme yield after optimization of immobilization and fermentation parameters (Table 12); and (e) increase in reusability of the 2.5% alginate beads from 11 cycles with less enzyme production compared lower alginate concentration beads (Table 1) to 26 cycles with enhanced protease production after optimization (Table 12).

Variations in bacterial cell biomass in the bead (Table 1) and observed differences in morphology and physiological behavior of immobilized *B. circulans* (Fig. 2c, g, and k) in different alginate concentration beads denote the importance of supramolecular structure of the matrix in substrate mass transfer and porosity of the gel beads. This shows the need to regulate the interaction of fermentation medium components and matrix concentrations with bacterial cells. The reduced cell number in 2.5% alginate beads may also be due to variations in mass transfer of various nutrients and other growth-required factors during fermentation, as mass transfer of nutrients, cellular products, and gases depend on the porosity of the immobilized bead. The substrate, products, and gas concentrations were known to be influential factors for microbial cell metabolism, which is also influenced by the immobilization matrix concentration and subsequent growth-associated cell number increase during fermentation [12, 16, 20]. The observed higher number of microbial cells per bead, prepared using 2.0% alginate concentration with respect to 2.5%, may also be due to decreased availability of the nutrients and other gases that, in turn, may lead to reduced cell growth inside the bead. Slight variation in enzyme productivity was observed with the change in matrix concentration (Table 1) and have been reported in literature [12, 16, 28, 29].

Extracellular enzyme yield with immobilized *B. circulans* cells was regulated by immobilization parameters, microenvironment, and fermentation medium component concentrations (Table 9 and 12). Optimization studies presented here further demonstrate

Table 12 Optimum conditions and their performance on alkaline protease production by immobilized *B. circulans* for L9 OA experiments.

S. no.	Factor	Level description	Level	Contribution
1	SA	2.50	2	16.601
2	Glucose	0.20	2	19.901
3	Incubation	18	3	09.997

Total contribution from all factors=46.499, current grand average of performance=52.415, expected results at optimum conditions=98.914, obtained 26 batches 5,680 units and 4.2×10^5 97.52

that each of the fermentation and immobilization parameters was important in the regulation of cellular-metabolism-associated enzyme production, as well as immobilized bead reusability either at individual or at interactive level. Similar trends were noticed with other enzyme production studies [10, 16, 20, 30]. The present study emphasizes the need of overall optimization with respect to higher reusability of immobilized beads, lower cell release into the medium, and higher enzyme production for economizing enzyme yield by immobilized cells.

Conclusion

Effective enzyme production by sodium-alginate-immobilized *B. circulans* cells mainly depends on the reusability of the immobilized beads, number of cells present in immobilized bead, and enzyme production in each fermentation cycle. All these are interlinked with concentration of immobilization matrix material, which has influence on mass transfer of substrate and products and fermentation medium component interactions with cell metabolism. A combination of factors and their levels involved in the economic production of protease by immobilized *B. circulans* were identified, and data were analyzed using the Taguchi OEC by assigning relative weightage to the reusability of immobilized beads, number of cell present in the medium during fermentation, and protease production. The OEC approach has proved to be efficient in the optimization of protease production. Glucose concentration has influenced significantly at individual level but played least influential role at interactive level. Overall, the enzyme productivity was improved from 214 to 315 U/ml per h under optimized fermentation environment with 2.5% sodium alginate immobilized *B. circulans*.

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