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Production of Alkaline Protease With Immobilized Cells of Bacillus subtilis PE-11 in Various Matrices by Entrapment Technique

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ABSTRACT

The purpose of this investigation was to study the effect of *Bacillus subtilis* PE-11 cells immobilized in various matrices, such as calcium alginate, k-Carrageenan, ployacrylamide, agar-agar, and gelatin, for the production of alkaline protease. Calcium alginate was found to be an effective and suitable matrix for higher alkaline protease productivity compared to the other matrices studied. All the matrices were selected for repeated batch fermentation. The average specific volumetric productivity with calcium alginate was 15.11 U/mL/hour, which was 79.03% higher production over the conventional free-cell fermentation. Similarly, the specific volumetric productivity by repeated batch fermentation was 13.68 U/mL/hour with k-Carrageenan, 12.44 U/mL/hour with agar-agar, 11.71 U/mL/hour with polyacrylamide, and 10.32 U/mL/hour with gelatin. In the repeated batch fermentations of the shake flasks, an optimum level of enzyme was maintained for 9 days using calcium alginate immobilized cells. From the results, it is concluded that the immobilized cells of *B subtilis* PE-11 in calcium alginate are more efficient for the production of alkaline protease with repeated batch fermentation. The alginate immobilized cells of *B subtilis* PE-11 can be proposed as an effective biocatalyst for repeated usage for maximum production of alkaline protease.

KEYWORDS: Alkaline protease production, *B subtilis* PE-11, immobilized cells, repeated batch fermentation.

INTRODUCTION

Proteases constitute one of the most important groups of industrial enzymes that are now used in a wide range of industrial processes, eg, in the detergent, food, pharmaceutical, leather, and silk industries.[1-6] With the exception of pharmaceutical uses, the detergent industry has emerged as one of the major consumers of hydrolytic enzymes working at alkaline pH, and now accounts for moe than a quarter of the global enzyme production.[7] At present, the use of alkaline proteases has increased remarkably with large proportions of commercially available alkaline proteases derived from *Bacillus* strains.[8,9]

Modification of biotechnology and processes, using immobilized biocatalysts, has recently gained the attention of many biotechnologists. Application of immobilized enzymes or whole cells is advantageous, because such biocatalysts display better operational stability[10,11] and higher efficiency of catalysis,[12,13] and they are reusable.

Microbial products are usually produced either by free or immobilized cells. The use of immobilized cells as industrial catalysts can be advantageous compared to batch fermentation process.[14,15] Whole cell immobilization has been a better choice over enzyme immobilization.[16,17]

Whole cell immobilization by entrapment is a widely used and simple technique. Romo and Perezmartinez[18] reported the viability of microbial cells over a period of 18 months under entrapped conditions and it was considered as one of the potential applications. The success achieved with the entrapment technique prompted us to study the production of alkaline protease with immobilized cells using this technique.

The purpose of the present investigation was to study the immobilization of *Bacillus subtilis* PE-11 cells for higher alkaline protease production using different entrapment techniques with matrices such as calcium alginate, k-Carrageenan, polyacrylamide, agar-agar and gelatin gel. The reusability of immobilized cells for alkaline protease production under repeated batch fermentation conditions was also investigated.

MATERIALS AND METHODS

Microorganism

An alkaline protease–producing strain of *B subtilis* PE-11 was isolated in our laboratory.[7] It was maintained on nutrient agar slants at 4°C and was subcultured every 4 weeks.

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Inoculum Preparation

Five milliliters of sterile distilled water was added to a 24-hours old slant of *B subtilis* PE-11. The cells were scrapped from the slant into sterile distilled water and the resulted cell suspension at 10% level was transferred, asceptically into 250-mL Erlenmeyer flasks containing 45 mL of sterile inoculum medium. The composition of the inoculum medium is (g/L): glucose, 2.0; casein, 0.5; peptone, 0.5; yeast extract, 0.5, and salt solution, 50 mL (salt solution containing [g/L]: KH_2PO_4, 5.0; $MgSO4.7H_2O$, 5.0, and $FeSO_4.7H_2O$, 0.1] with a pH of 7.0. The flask was kept in a shaker incubator at 220 rpm at 37ºC. The content of the flasks was centrifuged at 3000 rpm for 10 minutes and the supernatant was decanted. The cell pellet was washed thoroughly with sterile 20.0 g/L potassium chloride solution, followed by sodium chloride solution and sterile distilled water subsequently. Finally the cell mass was suspended in sterile sodium chloride solution (9.0 g/L). This cell suspension was used as inoculum for immobilization as well as for free-cell fermentations.

Whole Cell Immobilization by Entrapment

The alginate entrapment of cells was performed according method of Johnsen and Flink.[19] Sodium alginate solution (3%) (Loba Chemie, Mumbai, India) was prepared by dissolving sodium alginate in 100 mL boiling water and autoclaved at 121°C for 15 minutes.

Both alginate slurry and cell suspension (equivalent to 0.03 g dry cell weight [DCW]) were mixed and stirred for 10 minutes to get a uniform mixture. The slurry was taken into a sterile syringe and added dropwise into 0.2 M $CaCl_2$ solution from 5-cm height and kept for curing at 4°C for 1 hour. The cured beads were washed with sterile distilled water 3 to 4 times. When the beads were not being used, they were preserved in 0.9% sodium chloride solution in the refrigerator. All operations were carried out aseptically under laminar flow unit.

Immobilization of Whole Cells in Polyacrylamide

A cell suspension was prepared by adding 0.03 g cells to 10 mL chilled sterile distilled water. To another 10 mL of 0.2 M sterile phosphate buffer (pH 7.0), the following chemicals were added: 2.85 g acrylamide (Fluka, Buchs, Switzerland), 0.15 g bisacrylamide (Fluka), 10 mg ammonium persulphate, and 1 mL TEMED (NNN^1N^1 tetra methyl ethylene diamine). The cell suspension and the above phosphate buffer mixture was mixed well and poured into sterile flat bottom 10 cm-diameter petriplates. After polymerization (solidification), the acrylamide gel was cut into equal size cubes (4 mm^3), transferred to 0.2 M phosphate buffer (pH 7.0), and kept in the refrigerator for 1 hour for curing. The cubes were washed 3 to 4 times with sterile distilled water and stored in sterile distilled water at 4°C until use.

Immobilization of Whole Cells in k-Carrageenan

k-Carrageenan (4%) (Sigma, Mumbai, India) was weighed and added to 18 mL of 0.9% sodium chloride. It was dissolved by gentle heating and sterilized by autoclave. The cell suspension (2 mL equivalent to 0.03 g DCW) was added to the molten k-Carrageenan solution maintained at 40°C, mixed well, and poured into sterile flat bottom 4-inch-diameter petriplates. After solidification, the k-Carrageenan blocks were cut into equal size cubes (4 mm^3) and added to sterile 2% potassium chloride solution and kept in the refrigerator for 1 hour for curing. The cubes were washed 3 to 4 times with sterile distilled water.[20]

Immobilization of Whole Cells in Agar-agar

A definite quantity of agar–agar (Hi-media, Mumbai, India) was dissolved in 18 mL of 0.9% sodium chloride solution to get final concentration of 2% and sterilized by autoclaving. The cell suspension (2 mL equivalent to 0.03 g DCW) was added to the molten agar-agar maintained at 40°C, shaken well for few seconds (without forming foam), poured into sterile flat bottom 4-inch-diameter petriplates and allowed to solidify. The solidified agar block was cut into equal size cubes (4 mm^3), added to sterile 0.1 M phosphate buffer (pH 7.0), and kept in the refrigerator (1 hour) for curing. After curing, phosphate buffer was decanted and the cubes were washed with sterile distilled water 3 to 4 times.[20]

Immobilization of Whole Cells in Gelatin

Five milliliters (0.06% DCW) of cell suspension was added to 15 mL of 20% sterile gelatin (Hi-media), maintained at 45°C, and poured into a sterile petridish. The gel was overlayered with 10 mL of 5% glutaraldehyde for hardening at 30°C. The resulting block was cut into small-size cubes (4 mm^3) and the cubes were washed thoroughly with sterile distilled water for complete removal of excess glutaraldehyde.[20]

Production of Alkaline Protease by Batch Process With Immobilized Cells

The immobilized beads/blocks (cells equivalent to 0.03 g DCW) were transferred into 50 mL of production medium in 250-mL Erlenmeyer flasks. The composition of production medium was (g/L): glucose, 5; peptone, 7.5, and salt solution, 5% ($MgSO_4.7H_2O$, 5 g/L; KH_2PO_4, 5 g/L; and

$FeSO_4.7H_2O$, 0.1 g/L) with a pH of 9.0. The flasks were incubated at 37°C for 48 hours. Samples were withdrawn at regular intervals of 6 hours and assayed for alkaline protease activity.

Production of Alkaline Protease by Repeated Batch Process

One of the advantages of using immobilized biocatalysts is that they can be used repeatedly and continuously. Therefore, the reusability of *B subtilis* cells immobilized in matrix was examined. After attaining the maximum production of alkaline protease (24 hours), the spent medium was replaced with fresh production medium (50 mL) and the process was repeated for several batches untill the beads/blocks started disintegrating. The enzyme titers and cell leakage of each cycle were determined.

Analytical Methods

Alkaline Protease Assay

The culture broth was centrifuged at 3000 rpm for 20 minutes and supernatant served as the crude enzyme source. Protease activity was assessed by the modified procedure based on the method of Tsuchida et al[21] using 2.0% casein in 0.2 M carbonate buffer (pH 10.0) as substrate. One unit of enzyme activity is defined as the amount of enzyme that released 1 μg of tyrosine/mL/min. All experiments were carried out in triplicate.

Cell Growth and Cell Leakage

Both cell growth in freely suspended cultures and cells leaked from the gel matrix were determined as cell dry weight by measuring the optical density at 600 nm. One absorbance unit was equivalent to 0.23 g/L (cell dry weight).

RESULTS AND DISCUSSION

Production of Alkaline Protease With Immobilized Cells in Various Matrices by Entrapment Techniques

Cell immobilization is one of the common techniques for increasing the overall cell concentration and productivity. The separation of products from immobilized cells is easier compared with suspended cell systems. Immobilization of cells may allow continuous operation of cultivation processes at high dilution rates. Last but not least, immobilization is a strategy for protecting cells from shear forces. Many different techniques for immobilizing cells have been proposed.[22-24]

Production of Alkaline Protease by Immobilized Cells in Calcium Alginate

The amount of cell mass entrapped in calcium alginate matrix increased gradually up to 24 hours of incubation after which there was no appreciable change (Figure 1), whereas with free cell fermentation gradual cell growth was observed up to 48 hours (Figure 2). There was a slight change in pH profiles due to changes in the metabolic activities of microorganisms.

The enzyme production was started at 6 hours with immobilized cells and reached a maximum level (486 U/mL) by 24 hours. On further incubation, enzyme production was gradually decreased, whereas maximum enzyme titer was observed by 48 hours in the case of free cells. It is evident that the alkaline protease production was higher with immobilized cells (486 U/mL) than that of free cells (405 U/mL). Ramakrishna et al[13] reported the immobilization of *Bacillus cereus* in calcium alginate and employed packed-bed and fluidized-bed reactors to continuously synthesize thermostable α-amylase. They spun alginate fibres by pultrusion technique to reduce the diffussional resistances in the gel matrix, and thereby a 24-fold increase in the productivity compared to batch fermentation with free-cells was attained.

Production of Alkaline Protease by Immobilized Cells in Polyacrylamide

Polyacrylamide was successfully used for immobilization of many enzyme systems.[25] It was also used for the immobilization of cells for the production of other primary metabolites.[26] A gradual increase in alkaline protease production was noticed from 6 hours onwards to 24 hours; on further incubation decline in alkaline protease titer was observed (Figure 3). The maximum alkaline protease titer of 347 U/mL was observed at 24 hours and it was found to be a lower titer compared to alginate matrix.

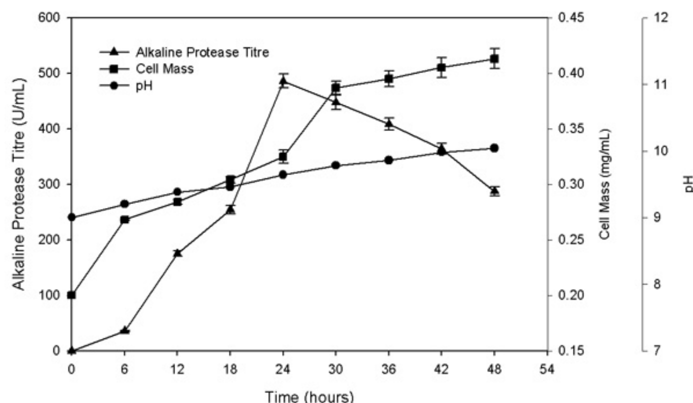

Figure 1. Time course profiles of pH, cell mass, and alkaline protease production by immobilized culture of *B subtilis* PE-11 in calcium alginate.

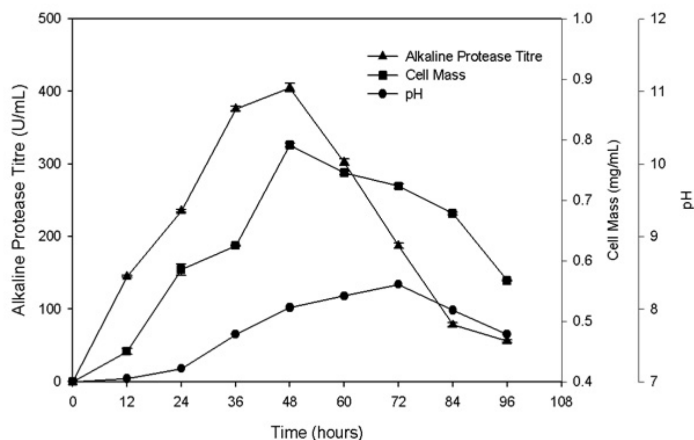

Figure 2. Time course profiles of pH, cell mass, and alkaline protease production by free cell culture of *B subtilis* PE-11.

Production of Alkaline Protease by Immobilized Cells in k-Carrageenan

A few reports on immobilization of *Streptomyces fradiae*[20] and *Penicillium chrysogenum*[27] cells for the production of tylosin and penicillin respectively were available in the literature about the use of k-Carrageenan as an entrapment matrix. The immobilization procedure is similar to alginate, and several other groups have used this polysaccharide as a preferred gel matrix either alone or in combination with other gums because of the mild conditions required and good gel stability. Using k-Carrageenan, immobilized *Brevibacterium flavum* attained high stability against several denaturing chemicals.[17] The rate of cell leakage could be lowered by hardening the gel with potassium cations.

The pattern of results is similar to Figure 3. The maximum alkaline protease titer (416 U/mL) was attained at 24 hours. The alkaline protease titer obtained with this carrier was less than that of free and immobilized cells with the other carrier (calcium alginate). There is negligible change in pH profile.

Production of Alkaline Protease by Immobilized Cells in Agar-agar

The results are similar to Figure 3 and the data indicated that alkaline protease production was started from 6 hours onward and reached a maximum level by 24 hours (358 U/mL). It was observed that the alkaline protease production with immobilized cells in agar-agar was less than the immobilized cells with other matrices (calcium alginate, polyacrylamide, and k-Carrageenan). The cell leakage from the matrix was gradually increased with increase of fermentation time. Anna et al[28] reported that the use of agar-entrapped cells of *Bacillus circulans* ATCC 21783 for cyclodextrin glucanotransferase production in a fluidized bed reactor led to enzyme activity (180 U/mL) after 24 hours of cultivation.

Production of Alkaline Protease by Immobilized Cells in Gelatin

The behavior of the results is similar to Figure 3. A detectable alkaline protease titer was observed at 6 hours of fermentation and reached a maximum level (302 U/mL) by 24 hours. The alkaline protease titer obtained with this carrier was very low compared with the titers of free cells and the immobilized cells of the above-mentioned other carriers. The natural polymers such as agar, agarose, pectin, and gelatin were also employed for cell immobilization.[17] They employed gel as a carrier material for the immobilization of *Kluyveromyces fragilis* for β-galactosidase activity and *Escherichia coli* for penicillin acylase.

Comparison of Alkaline Protease Production by Immobilized Cells in Various Matrices by Entrapment Technique

The alkaline protease titer with immobilized cells in alginate matrix was found to higher followed by k-Carrageenan (Table 1). Low level of alkaline protease production was observed with polyacrylamide and gelatin. Probably both glutaraldehyde (used for cross-linking with gelatin) and polyacrylamide monomers were toxic for the cells. A low level to moderate titer of alkaline protease was obtained with agar, and also it was proved to be unstable resulting in a considerable amount of cell leakage.

Repeated Batch Fermentation With Free Cells and Immobilized Cells

The semi-continuous fermentation was terminated to investigate the stability of the biocatalysts and their ability to produce alkaline protease under repeated batch cultivation conditions.

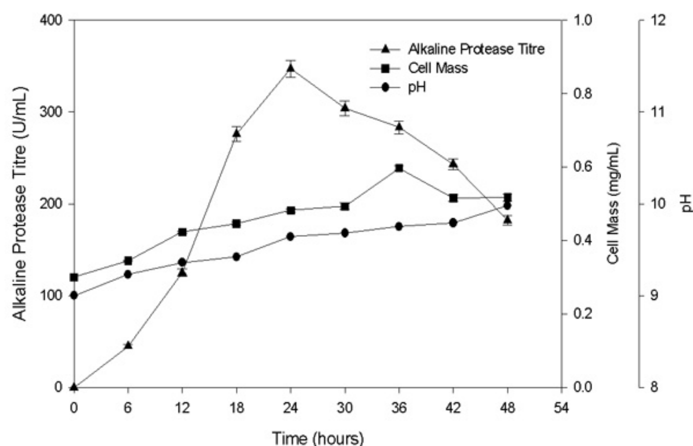

Figure 3. Time course profiles of pH, cell mass, and alkaline protease production by immobilized culture of *B subtilis* PE-11 in polyacrylamide.

Table 1. Comparison of Alkaline Protease Production Using *B subtilis* PE-11 Cells Entrapped in Various Gel Matrices After 24 hours of Fermentation

	Support Matrix	Final pH	Alkaline Protease Titre (U/mL)	Relative Percentage of Production
1.	Calcium alginate	9.64	486	100
2.	k-Carrageenan	9.54	416	85.59
3.	Agar-agar	9.62	374	76.95
4.	Polyacrylamide	9.64	347	71.39
5.	Gelatin	9.45	302	62.14
6.	Free cells	10.02	405	83.33

Figure 4 shows the possibility for reuse of the calcium alginate biocatalysts to produce alkaline protease in semicontinuous mode. The results revealed that the amount of enzyme production with types of immobilized cells gradual decrease in alkaline protease titer from the first batch onward was observed. At the same time the gradual cell leakage from the gel was observed from the first to the ninth batch. The beads were disintegrated during the 10th batch operation. Thus the repeated batch fermentation with calcium alginate beads was successfully run for 9 batches (9 days). These findings were in accordance with those obtained previously for the protease production by immobilized *Serratia marcescens* and *Myxococcus xanthus* in calcium alginate beads.[29] It was found that protease production by immobilized *S marcescens* increased with repeated growth cycles, and reached a maximum after 5 cycles. Bandyopadhyay et al[30] studied erythromycin production by *Streptomyces erythreus* entrapped in calcium alginate beads and obtained efficient productivity of erythromycin. They could conduct repeated batch fermentation successfully (each batch 48 hours) for 12 batches (30 days). Similarly, Farid et al[31] reported that a good level of oxytetracycline was produced for a period of 28 days (7 batches) using *Streptomyces rimosus* cells immobilized in 4% calcium alginate.

The immobilized cells entrapped in k-Carrageenan, agar-agar, polyacrylamide, and gelatin were also used for repeated batch fermentation. The behavior for these systems was similar to alginate-immobilized cells (Figure 4). With all these systems, relatively low enzyme titers were observed when compared to alginate-immobilized cells. Also, the cell leakage from the respective gels was more and the gel cubes disintegrated after 6 batches of fermentation.

Audet et al[32] suggested a cell entrapment process using k-Carrageenan and locust bean gum, which significantly modified the mechanical properties of the gel. Using the above mixed gel, several studies were carried out with various lactic acid–producing microorganisms.[33,34] The k-Carrageenan locust-bean-gum-mixed gel matrix showed significant stability for 3 months in continuous fermentation in a stirred-tank reactor. Continuous production of glucoamylase by immobilizing mycelial fragments of *Aspergillus niger* was demonstrated.[17] Among the several polymer matrices tried for immobilization, k-Carrageenan and alginate were the most effective. The authors reported the operation of aerated packed-bed reactor for a period 360 hours, attaining maximum productivity of 1638 IU/L/hour. The operational stability of the biocatalysts (agar-immobilized *Bacillus circulans* cells) for cyclodextrin glucanotransferase production was studied[28] by repeated batch cultivation for 240 hours (in a fluidized bed reactor).

Comparison of Alkaline Protease Production With Immobilized Cells in Various Matrices by Repeated Batch Fermentation

The data show that the average specific volumetric productivity with calcium alginate was 15.11 U/mL/hour, which was 79.03% higher production over the conventional free-cell fermentation (Table 2). Similarly, the specific volumetric productivity by repeated batch fermentation was 13.68 U/mL/hour with k-Carrageenan, 12.44 U/mL/hour with agar-agar, 11.71 U/mL/hour with polyacrylamide, and 10.32 U/mL/hour with gelatin.

The alginate matrix was found to be superior to the other matrices studied in this paper. In addition, the alginate matrix is less expensive, nontoxic, and preparation of biocatalyst involves mild conditions, which is an added advantage. In contrast, free cells showed lower enzyme productivity than the calcium alginate–immobilized ones and their activity decreased markedly with repeated batch cycles (data not shown). After the immobilized biocatalyst

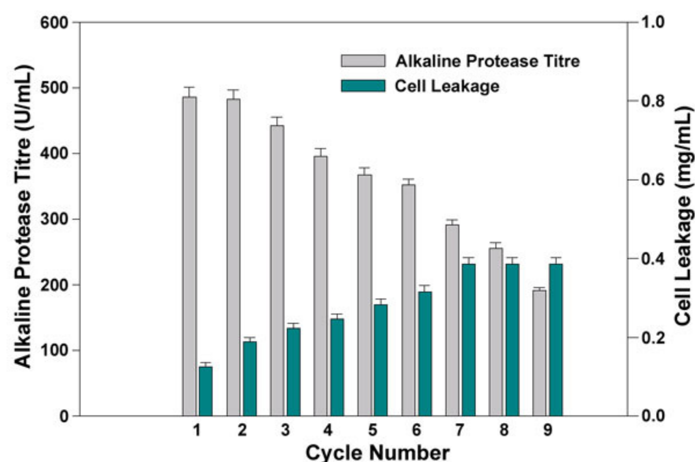

Figure 4. Alkaline protease production by repeated batch culture using immobilized cells of *B subtilis* PE-11 in calcium alginate.

Table 2. Comparison of Alkaline Protease Production With Cells Immobilized in Various Matrices by Repeated Batch Cultures

	Matrix	Fermentation Period for Each Batch (hours)	No. of Batches	Total Fermentation Time (hours)	Total Alkaline Protease Titre (U/mL)	Average Alkaline Protease Activity per Batch (U/mL)	Specific Volumetric Productivity (U/mL/hour)
1.	Calcium alginate	24	9	216	3264	362.66	15.11
2.	k-Carrageenan	24	6	144	1970	328.33	13.68
3.	Agar-agar	24	6	144	1792	298.67	12.44
4.	Polyacrylamide	24	6	144	1686	281.00	11.71
5.	Gelatin	24	6	144	1486	247.67	10.32
6.	Free cells (conventional)	48	1	48	405.00	405.00	8.44

Medium volume: 50 mL.

had been in use for about 9 days, it still possessed significant alkaline protease production. It has been shown that immobilized cells were able to produce alkaline protease consistently and that they might be used for continuous alkaline protease production.[5]

CONCLUSIONS

The results show that calcium alginate is a promising method of *B subtilis* PE-11 immobilization for alkaline protease production. Alkaline protease production by immobilized cells is superior to that of free cells because it leads to higher volumetric activities within the same time of fermentation. Specific advantages of this technique such as long life-term stability, reusablility, and possibility of regeneration to be adaptable also to scale-up the obtained data. In addition, the experiments with repeated batches of alginate immobilized bacterial growth by introducing fresh nutrients every 24 hours leads to a specific volumetric productivity that is 1.8 times (180%) higher than that obtained with free cells.

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