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Enantioselective Resolution of γ-Lactam by a Whole Cell of *Microbacterium hydrocarbonoxydans* (L29-9) Immobilized in Polymer of PVA–Alginate–Boric Acid

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Abstract Using immobilized cells of a novel strain of *Microbacterium hydrocarbonoxydans* L29-9 in polymers of polyvinyl alcohol (PVA)–alginate–boric acid, enantioselective resolution of racemic γ-lactam to produce (−)γ-lactam was successfully carried out. A 6:1 ratio of PVA:sodium alginate not only prevented agglomeration of the matrix but also produced beads with high gel strength. The optimum biotransformation conditions were 1 g/L substrate, pH 7.0, reaction temperature of 30 °C, and reaction time of 3 h. After every two cycles, the immobilized cell beads were separated and immersed in 0.5 mM KCl solution at 4 °C for preservation. At optimum conditions, the enantiomeric excess and the yield of (−)γ-lactam were >99% and 34%, respectively. The beads showed a slight decrease in the enantiomeric excess when re-used up to 14 cycles (the enantioselectivity of the immobilized cells decreased slightly after 14 cycles of usage).

Keywords Abacavir · Enantioselective resolution · γ-Lactam · Immobilized *Microbacterium hydrocarbonoxydans* cells · Polyvinyl alcohol

Introduction

Biocatalysis is one of the most important fields in biological technology due to its high enantioselectivity, mild reaction conditions, and low energetic consumption. It has been applied widely in the asymmetric synthesis for many chiral intermediates [1]. (−)2-Azabicyclo[2.2.1]hept-5-en-3-one ((−)γ-lactam) is a key intermediate for the synthesis of (−)abacavir, which serves as a powerful antiviral agent [2]. In preparation of (−)γ-lactam, biocatalysis methods are often employed [3–8].

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The whole cells of a novel strain (*Microbacterium hydrocarbonoxydans* L29-9) which produced γ-lactamase displayed excellent capabilities for the enantioselective resolution of (*rac*)γ-lactam (Fig. 1). However, a frequently occurring problem in biocatalytic processes with free cells or enzyme is the difficulty in reusing the biocatalyst. Immobilized cells can be re-used, and the costs of extra addition of cofactors and purification of enzymes can be avoided. There are many applications of immobilized cells on different matrices for biocatalysis transformation [9–12].

Polyvinyl alcohol (PVA) is one of the most widely used immobilization materials. PVA is a raw material of vinylon and can be economically produced. Furthermore, immobilization with PVA gel has been frequently adopted due to its advantages over other gels as a carrier for cells [13–15].

In the present work, employing PVA as a gel matrix for immobilization of *M. hydrocarbonoxydans* was conducted. The aim of the work was to produce (−)γ-lactam using the biocatalysis method, employing immobilized cells in batch mode. After optimizing the immobilization conditions, the effects of pH, reaction temperature, reaction time, and substrate concentration were tested in order to further optimize the resolution process. Optimization of the preservation method was also conducted.

Experiment

Chemical Materials

γ-Lactam and *N*-acetylphenylalanine were obtained from Sigma (Germany). All organic solvents of HPLC grade were purchased from Acros (USA). Unless otherwise stated, all reagents were of analytical grade and were purchased from Beijing Chemical Co. (People's Republic of China).

Strain and Culture Conditions

M. hydrocarbonoxydans L29-9 strain was conserved in our lab.

M. hydrocarbonoxydans L29-9 was maintained on an agar slant (tryptone 10 g/L, yeast extract 5.0 g/L, NaCl 10 g/L, *N*-acetylphenylalanine 3.0 g/L, and agar 20 g/L, pH 7.0).

Initially, a seed culture was developed by inoculating L29-9 into 5 mL sterilized liquid culture medium (yeast extract 5.0 g/L, Na_2HPO_4 7.0 g/L, KH_2PO_4 2.0 g/L, $MgSO_4$ 0.4 g/L, citric acid 2.0 g/L, $FeSO_4$ 0.008 g/L, $CaCl_2$ 0.01 g/L, and *N*-acetylphenylalanine 3.0 g/L, pH 7.0) and incubated in a rotary shaker for 24 h at 220 rpm, 30 °C. The cells were then transferred into a 250 mL Erlenmeyer flask containing 40 mL liquid culture medium and

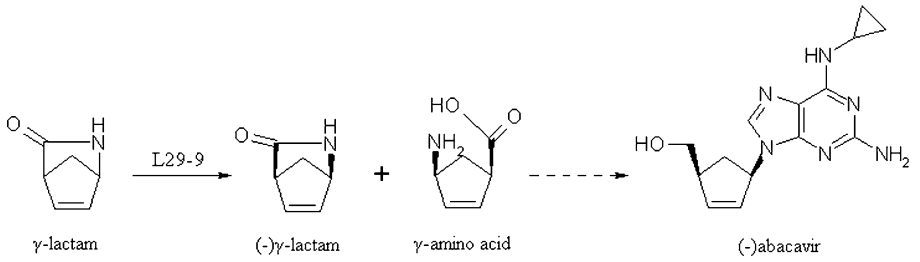

Fig. 1 Enantioselective resolution of (*rac*)γ-lactam by *Microbacterium hydrocarbonoxydans* L29-9 to synthesis of (−)abacavir

incubated in a rotary shaker for 24 h at 220 rpm, 30 °C. The cells were harvested by centrifugation (12,000×*g* for 3 min) and washed with phosphate buffer (0.05 mM, pH 7.0).

Immobilization of Whole Cells Using the PVA–Alginate–Boric Acid Method

A traditional external gelation method [16, 17] was employed in this work.

For immobilization, 0.1 g sodium alginate and 0.6 g PVA were dissolved in 10 mL distilled water by being heated to 121 °C. The solution was then cooled to 35 °C, followed by addition of 1 mL of cell suspension (175 mg dry weights—dry cell weight was determined by drying at 80 °C, until constant weight—were suspended to 1.0 mL of 0.05 M phosphate buffer (pH 7.0)) and mixed thoroughly.

Using a syringe to form spherical beads, the mixture was then dropped into a gently stirred ice cold 3% boric acid solution containing 2% $CaCl_2$ (pH 6.7). In order to complete gelation inside the beads, they were kept in boric acid solution for 1 h at 4 °C. The beads were then collected and washed three times with sterile distilled water.

Optimization of the Batch Biotransformation Conditions

Batch resolutions were carried out in 250 mL Erlenmeyer flasks containing 4.5 g of immobilized cells in 20 mL 1 g/L γ-lactam aqueous solution. The flasks were maintained in an orbital shaker at 220 rpm 30 °C for 3 h. At the end of each run, the reaction medium was removed by filtration, and the immobilized cells were re-fed with fresh substrate to investigate the stability of immobilized cells. The enantioselectivity of each run was analyzed.

Optimization of the reaction conditions is necessary to ensure the highest enantioselectivity of the immobilized cells. The biotransformation was carried out at a different pH and temperature. Different concentrations of the γ-lactam solution and biotransformation times were tested.

Preservation of Immobilized Cells

After every two batch biotransformations, the immobilized cells were collected by filtration and washed with distilled water three times. The immobilized cells were then immersed in preservation reagent. Different preservation reagents including phosphate buffer (pH 7.0), saline solution (0.9%), Tris–HCl buffer (pH 7.5), acetate buffer (pH 5.8), deionized water, and glycerol (15%, *v*/*v*) were tested. The effect of 1 mM metal ion on preservation was analyzed, including Na^+, K^+, Mg^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, Ca^{2+}, and Cu^{2+}.

Analytical Methods

The enantioselectivity for the biotransformation was expressed by enantiomeric excess (*e.e.S*) [18]:

$$e.e.S = S_+ - S_-/S_+ + S_- \times 100\%$$

The product yield was determined from the ratio of the amount of produced (−)γ-lactam to the amount of the initial racemic γ-lactam,

$$\text{yield} = S_-/S_0 \times 100\%,$$

where S_+, S_- and S_0 stand for (+)γ-lactam (−)γ-lactam and racemic γ-lactam, respectively.

e.e.S and yield were determined by Chiral-HPLC (LC-20AT, Shimadzu Corporation, Kyoto, Japan). The reaction mixture was extracted with 1 mL ethyl acetate. Ethyl acetate

extract (10 μL) was applied to a DAICEL CHIRALPAK AS-H column (4.6 mm diameter, 250 mm long, 5 μm film) and was eluted with a mobile phase (80% acetonitrile, 20% isopropanol; *v*/*v*). The UV absorption of γ-lactam was monitored at 230 nm. Benzamide (internal standard) and (+) and (−)γ-lactam have retention times of 8.7, 11.8, and 13.9 min, respectively, at a flow rate of 0.6 mL/min.

Results and Discussion

Optimization of Immobilization Conditions

Effect of Matrix Concentration

A series of ratios of PVA to sodium alginate were tested. It was found that a 6:1 ratio of PVA to sodium alginate not only prevented agglomeration but also produced a gel with significant strength and a regular spherical shape. The resulting spherical beads were highly elastic and had an average diameter of 3.0 mm. It was difficult to produce spherical gel beads using lower ratios of PVA. Ratios of PVA higher than 6:1 resulted in a viscous solution and made the immobilization process very difficult.

Effect of Cell Concentration

The enantiomeric excess was found to increase with increasing cell concentrations. However, the cells leaked from the matrices when its concentration exceeded 175 mg/mL (Fig. 2).

Time Course for Embedment

After spherical gel beads of immobilized cells were formed, in order to complete gelation inside beads, these beads were kept in boric acid solution for different times at 4 °C and

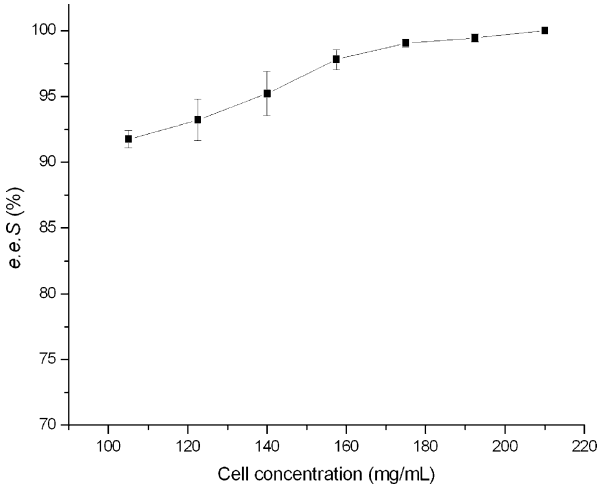

Fig. 2 Effect of cell concentration for embedment. Reaction conditions: γ-lactam concentration, 1 g/L; reaction time, 3 h; temperature, 30 °C; pH, 7.0; reaction medium volume, 20 mL. *Squares*, *e.e.S*

then used for biotransformation. The immobilized cells that were embedded for 1 h gave the highest activity and enantiomeric excess (Fig. 3).

Embedding of the immobilized beads with the boric acid greatly affects gel strength [16]. However, because of the toxicity of boric acid, it was thought that enzyme activity would be lost during a long period of embedding.

Optimization of Enantioselective Resolution Conditions

Effect of pH on Enantioselective Resolution

Efficient resolution of γ-lactam was observed when the pH was maintained at levels from 5.0 to 10.0 by the immobilized cells. The optimal pH was 7.0 with >99% enantiomeric excess of (−)γ-lactam (Fig. 4).

Effect of Temperature on Enantioselective Resolution

The temperature profile in the range of 24–45 °C was examined for the product of (−)γ-lactam. As shown in Fig. 5, higher temperatures was more detrimental to γ-lactam resolution than lower temperatures. However, the enantioselectivity of the reduction was not significantly influenced in reaction temperatures from 25 °C to 35 °C. The optimum temperature range was 25–35 °C for the enantiomeric excess of the desired product (−)γ-lactam.

Effect of γ-Lactam Concentration on Enantioselective Resolution

In order to find the optimum concentration of substrate γ-lactam, several levels of substrate γ-lactam concentrations from 0.5 to 6.0 g/L were presented. As shown in Fig. 6, the optimum concentration of the substrate was 1.0 g/L with >99% enantiomeric excess of

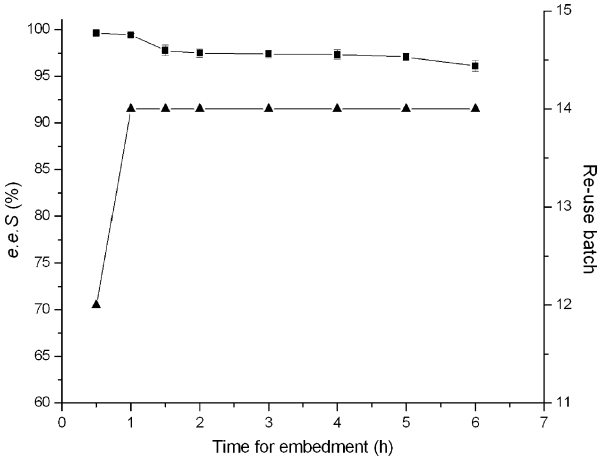

Fig. 3 Effect of time for embedment. Reaction conditions: cell concentration, 175 mg/mL; γ-lactam concentration, 1 g/L; reaction time, 3 h; temperature, 30 °C; pH, 7.0; reaction medium volume, 20 mL. *Squares*, *e.e.S*; *triangles*, re-use batch

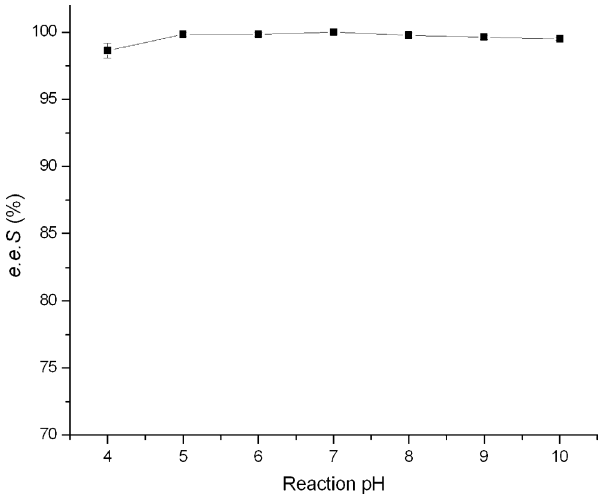

Fig. 4 Effect of pH on enantioselective resolution. Reaction conditions: cell concentration, 175 mg/mL; γ-lactam concentration, 1 g/L; reaction time, 3 h; temperature, 30 °C; reaction medium volume, 20 mL. *Squares*, *e.e.S*

(−)γ-lactam. If the concentration of γ-lactam was above 1.0 g/L, the activity of immobilized cells decreased, probably because substrate inhibition had occurred.

Time Course of Enantioselective Resolution

The time period for the enantioselective resolution of γ-lactam is depicted in Fig. 7. The enantiomeric excess increased for 3 h until reaction and then decreased. The optimum time on enantioselective resolution was 3 h with the enantiomeric excess of (−)γ-lactam >99%.

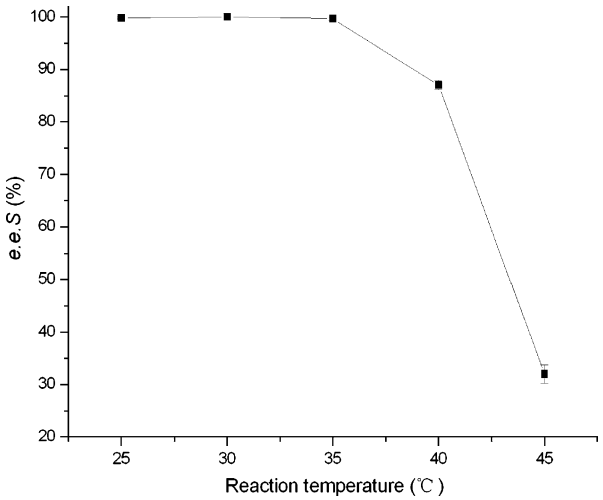

Fig. 5 Effect of temperature on enantioselective resolution. Reaction conditions: cell concentration, 175 mg/mL; γ-lactam concentration, 1 g/L; reaction time, 3 h; pH, 7.0; reaction medium volume, 20 mL. *Squares*, *e.e.S*

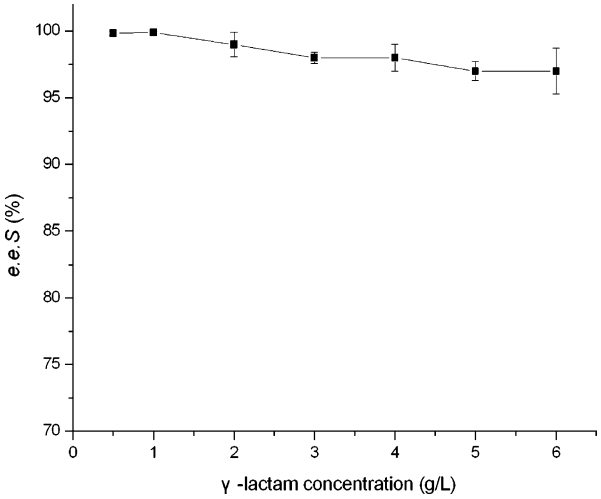

Fig. 6 Effect of γ-lactam concentration on enantioselective resolution. Reaction conditions: cell concentration, 175 mg/mL; reaction time, 3 h; temperature, 30 °C; pH, 7.0; reaction medium volume, 20 mL. *Squares*, *e.e.S*

Optimization of Preservation Conditions

Optimization of Preservation Reagents

Immobilized cells beads that were immersed in phosphate buffer (pH 7.0), Tris–HCl (pH 7.5), acetate buffer (pH 5.8), or glycerol (15%, *v*/*v*) were disrupted after 12 h preservation at 4 °C. Hydroxyl in these buffers can react with calcium alginate, thus

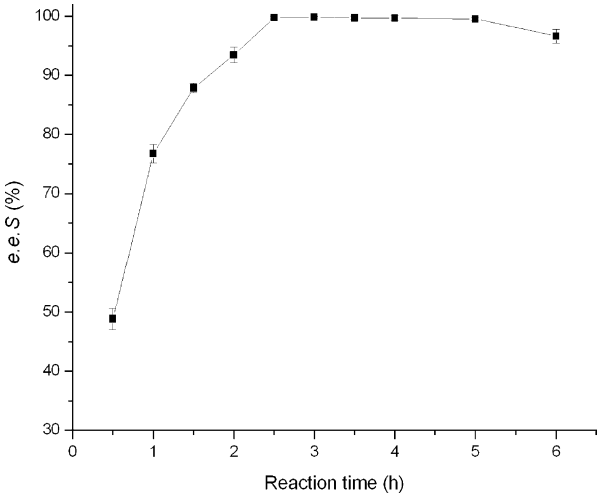

Fig. 7 Optimization of reaction time on enantioselective reduction. Reaction conditions: cell concentration, 175 mg/mL; γ-lactam concentration, 1 g/L; temperature, 30 °C; pH, 7.0; reaction medium volume, 20 mL. *Squares*, *e.e.S*

causing the disruption of the immobilized cells. The immobilized cells beads preserved in 0.9% physiological saline and deionized water were kept intact and the latter preservative buffer gave higher enantiomeric excess (>99%) compared to the former.

Effect of Metal Ions

Usually, metal ions play a significant role on the cell growth and metabolite biosynthesis because of their involvement in metalloenzymes or as enzyme activators. Metal ions fill the role of a bridge linking the enzyme with substrate, thus facilitating the combination of substrates with enzyme active centers. The effect of metal ions toward enzymes have a certain selectivity, that is, one kind of metal ion may have an activation effect on some enzymes but an inhibitory activity on others. Effect of different metal ions on immobilized L29-9 cells in terms of enantiomeric excess and re-used batches are shown in Fig. 8. Na^+, K^+, and Mg^{2+} could enhance the enantiomeric excess of (−)γ-lactam, while Fe^{2+}, Mn^{2+}, Ni^{2+}, Ca^{2+}, and Cu^{2+} showed negative effects. A maximal enantiomeric excess (97%) was obtained by adding 1 mM KCl to preserving solution, and in this buffer, the maximal reusability was 14 batches.

Effect of different K^+ concentrations on preservation was tested. The enantiomeric excess remained constant, regardless of various concentrations of K^+ that were added into

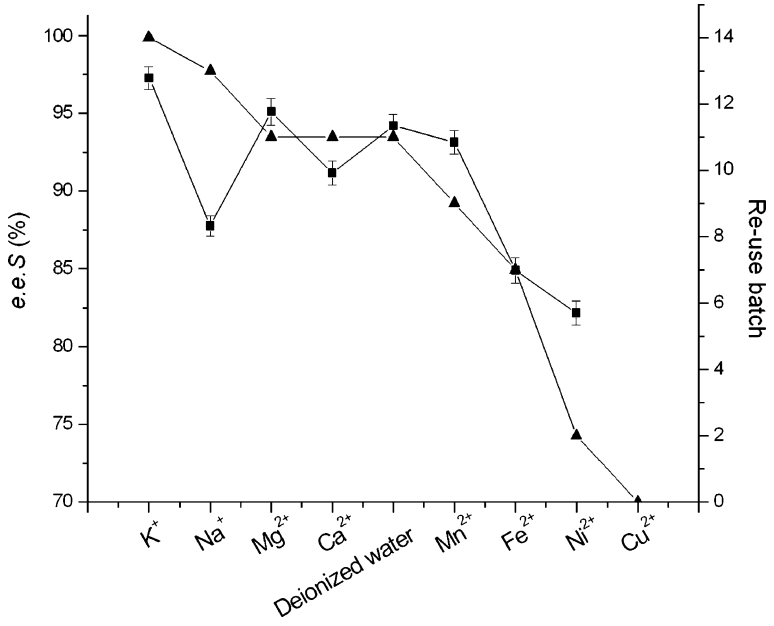

Fig. 8 Effect of metal ions on immobilized cells. Reaction conditions: cell concentration, 175 mg/mL; γ-lactam concentration, 1 g/L; reaction time, 3 h; temperature, 30 °C; pH, 7.0; reaction medium volume, 20 mL. *Squares*, *e.e.S*; *triangles*, re-use batch

the preservation buffer. This indicated that there was no significant effect of the K^+ concentration on the preservation of immobilized cells. For economical consideration, 0.5 mM K^+ was employed for immobilized cells preservation.

Operation Stability of Immobilized L29-9 Cells

The operational stability of immobilized cells, without a large loss of enzyme activity, is important for biosynthetic processes. To investigate the operation stability of immobilized cells, the immobilized cells were repeatedly used for transformation. After each batch, the beads were separated by filtration and washed with distilled water three times; the immobilized cells were then collected and immersed in a fresh transformation buffer. Only after ten batches of transformation did activity and enantioselectivity of the immobilized cells decrease slightly (Fig. 9).

Conclusions

The PVA–alginate–boric acid method is an efficient method for immobilization of L29-9 cells. Most importantly, immobilized cells can be conveniently used in repeated batch biotransformations. In our case, under optimum conditions, immobilized cells can be used at least for ten batches of transformation with only a slight decrease in activity and enantioselectivity. Therefore, this is quite a promising method for industrial application of resolution of γ-lactam.

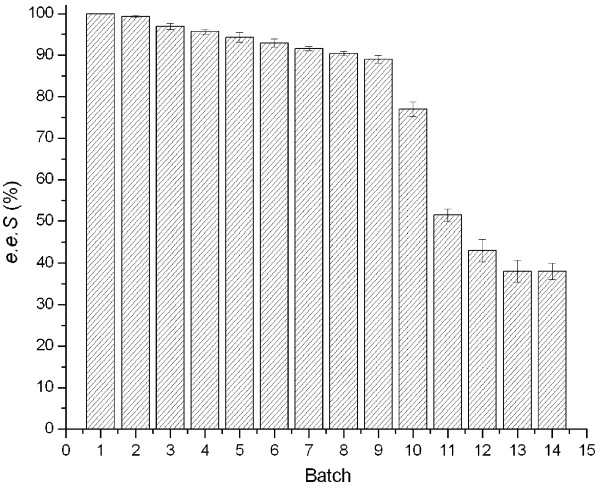

Fig. 9 Operation stability of immobilized L29-9 cells. Reaction conditions: cell concentration, 175 mg/mL; γ-lactam concentration, 1 g/L; reaction time, 3 h; temperature, 30 °C; pH, 7.0; reaction medium volume, 20 mL. *Diagonal hatched bars*, *e.e.S*

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