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Efficient production of hyaluronic acid by *Streptococcus zooepidemicus* using two-stage semi-continuous fermentation

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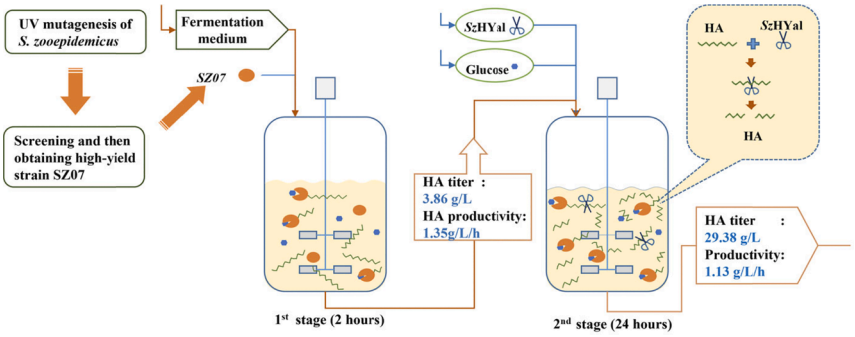
HIGHLIGHTS

- Two-stage semi-continuous fermentation was developed for hyaluronic acid production.
- High hyaluronic acid titer and productivity can be attained in 2nd stage bioreactor.
- Addition of hyaluronidase can reduce viscosity of broth and improve production.

GRAPHICAL ABSTRACT



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ABSTRACT

Hyaluronic acid is a kind of mucopolysaccharide that has wide applications in cosmetics, health food, and orthopedics. Using *Streptococcus zooepidemicus* ATCC 39920 as parent, a beneficial mutant SZ07 was obtained by UV mutagenesis, giving 1.42 g/L hyaluronic acid in shake flasks. To enhance the efficiency of hyaluronic acid production, a semi-continuous fermentation process consisted of two-stage 3–L bioreactors was developed, in which 1.01 g/L/h productivity and 14.60 g/L hyaluronic acid were obtained. To further enhance the titer of hyaluronic acid, recombinant hyaluronidase *Sz*HYal was added into 2nd stage bioreactor at 6 h to reduce the viscosity of broth. The highest hyaluronic acid titer of 29.38 g/L was achieved with a productivity of 1.13 g/L/h at 300 U/L *Sz*HYal after 24 h. This newly developed semi-continuous fermentation process provides a promising strategy for the industrial production of hyaluronic acid and related polysaccharides.

1. Introduction

Hyaluronic acid (HA) is a long-chain mucopolysaccharide formed by repeating disaccharide units consisted of *N*-acetylglucosamine and glucuronic acid (Yao et al., 2021). HA mainly exists in the chicken combs, eyes, umbilical cords and joints (Zhang et al., 2021). Among various microorganisms, HA is mainly produced by *Streptococcus zooepidemicus*, and often secreted and wrapped on the surface of cells (Wessels et al., 1991). HA has a wide range of molecular weight (Mw) from thousands to millions of Daltons (Da), which corresponds to different functions

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(Rossatto et al., 2023). HA has versatile applications in the medicine, beauty, and food industries (El-Aassar et al., 2020; Saha and Rai, 2021; Sutariya and Salunke, 2022; Zhang et al., 2022). For example, HA with Mw of over10 kDa is useful in cosmetic and orthopedic treatments, while HA of less than 5 kDa can inhibit inflammation and promote angiogenesis (Allison and Grande-Allen, 2006; de Mera et al., 2019; Fagien and Cassuto, 2012).

Currently, HA has been authorized as a food ingredient in the United States, Canada, China, Japan, South Korea, Italy, and Belgium, undoubtedly a huge market in the future (Oe et al., 2014; Sutariya and Salunke, 2022). There are two major production methods for HA: microbial fermentation and animal tissue extraction (Oregan et al., 1994; Yao et al., 2021). HA production strains mainly include *S. zooepidemicus*, *Corynebacterium glutamicum*, and *Bacillus subtilis*. Currently, the industrial production of HA mainly depends on the fed-batch fermentation of *S. zooepidemicus*. Additionally, several recombinant microbial strains have been constructed for the heterogeneous synthesis of HA (Blank et al., 2005; Jia et al., 2013). In microbial fermentation, the accumulation of HA is often hampered by the high viscosity of culture broth (Chong et al., 2005). In fed-batch fermentation using engineered *C. glutamicum* (Wang et al., 2020) and *B. subtilis* (Jin et al., 2016), recombinant hyaluronidase originated from leech was supplemented to reduce the viscosity of broth and enhance HA production, and HA titers were 74.1 g/L and 19.38 g/L, respectively. Continuous fermentation using *S. equi* subsp. *zooepidemicus* ATCC 35246 resulted in a HA titer of 0.66 g/L with a productivity of 0.26 g/L/h (Blank et al., 2005). In batch fermentation by *Streptococcus* sp. ID9102, 6.94 g/L HA was attained by optimizing the fermentation conditions (Im et al., 2009). By knocking out phosphofructokinase and overexpression of sucrose-6-phosphate hydrolase in *S. zooepidemicus* ATCC 39920, a HA titer of 5.6 g/L was obtained in batch fermentation (Zhang et al., 2016). In fed-batch fermentation, the use of *Streptomyces albulus* resulted in a HA titer of 6.2 g/L by strengthening the synthetic pathway and weakening the competitive pathways (Yoshimura et al., 2015). In fed-batch fermentation, HA titer of 6.09 g/L was reached by *Lactococcus lactis* CES15 overexpressing hyaluronan synthase (Sunguroglu et al., 2018).

Compared with *C. glutamicum* and *B. subtilis*, the fermentation period of *S. zooepimicus* is relatively shorter (within 24 h), and the product recovery is more convenient due to its relatively lower cell density. However, the application of *S. zooepimicus* in the production of HA is often hindered by several issues. (i) HA titer and productivity of *S. zooepimicus* are relatively lower than those of *C. glutamicum* and *B. subtilis* (Qiu et al., 2021). (ii) Production of HA is severely limited by the low cell density of *S. zooepimicus* (<6.0 OD_{600}) (Ucm et al., 2022). In this study, fermentation conditions were optimized to promote the cell growth of *S. zooepidemicus*, and the productivity of *S. zooepidemicus* was enhanced using a two-stage semi-continuous fermentation process. Furthermore, the titer of HA was increased by addition of hyaluronidase in the 2nd-stage bioreactor during semi-continuous fermentation.

2. Materials and methods

2.1. UV mutagenesis of Streptococcus zooepidemicus ATCC 39920

S. zooepidemicus ATCC 39920, capable of producing HA, was purchased from Shanghai Huike Biotechnology (Shanghai, China). The strain was subjected to UV mutagenesis for enhanced production of HA. *S. zooepidemicus* ATCC 39920 was cultured to OD_{600} of 0.4 in 250-ml shake flasks containing 20 ml Luria-Bertani (LB) medium, washed three times with sterile saline solution and dispersed in 10 ml deionized distilled water. Then, the above prepared cell suspension (5 ml) was added into a 9-cm sterile petri dish with a magnetic stir bar for stirring at 50 rpm. The plate was irradiated for 50–150 s at a distance of 30 cm from the UV lamp (30 W, UV-C). The lethal rate was controlled at 70–80 %. After irradiation, the cell suspension was diluted 10^4–10^6 times with sterile saline, and 100 μL of diluted cell solution was spread on LB agar plates. Three replicates were performed for each irradiation condition, and the plates were wrapped in black plastic bags and incubated at 37 °C. The above operations were performed under red light. When colonies were observed, 5 ml of bovine serum albumin solution (50 g/L) was added to the plate, and then 10 % glacial acetic acid was added for 20 min incubation. Since the mixture of bovine serum albumin and HA will produce white precipitation, colonies with significantly increased white precipitation circles were selected for the next round of screening. Finally, the isolated strains were inoculated into 500-mL shake flasks for fermentation and HA production analysis.

2.2. Recombinant expression of hyl from Streptococcus zooepidemicus

E. coli JM109 was used for recombinant plasmid construction and amplification. *E. coli* BL21 (DE3) was used for recombinant expression. Gene *hyl* (GenBank No: EU082206) encoding hyaluronidase was amplified from the genomic DNA of *S. zooepidemicus* using primers *hyl*-F (5′- tatcggaattaattcggatccATGGCAACAGGAACTGAG-3′) and *hyl*-R (5′-ctcgagtgcggccgcaagcttCTATGATAAGGCCTTAAAAG-3′). Then, *hyl* was inserted into pET-22b with signal peptide *pelB* using restriction sites *BamH*I and *Hind*III to construct recombinant plasmid pET-22b-*hyl*.

E. coli BL21 (DE3) harboring pET-22b-*hyl* was cultivated at 37 °C in LB medium containing 100 μg/L ampicillin. After inoculation with 1 % (v/v) seed culture, 0.1–0.5 mM isopropyl-β-D-thiogalactoside (IPTG) and 7.5 g/L glycine were added to induce the expression of *hyl* at OD_{600} of 0.8–1.2. Then, the culture was further induced at different temperatures (16–30 °C) for 48 h to produce recombinant *Sz*HYal (Nie et al., 2013).

The activity of hyaluronidase *Sz*HYal was determined following colorimetric method based on 3,5-dinitrosalicylic acid, in which the reducing sugar was monitored (Huang et al., 2020).

2.3. Batch fermentation by Streptococcus zooepidemicus

Batch fermentation was performed in 3–L bioreactors containing 1.5 L medium (80 g/L initial glucose) (Rangaswamy and Jain, 2008). The seed culture of *S. zooepidemicus* was cultured in sucrose-peptone medium containing 20 g/L sucrose and 10 g/L peptone 205 at 37 °C and 200 rpm for 10 h. Then, 150 ml seed culture (10 % inoculum) was inoculated into a 3–L bioreactor containing 1.35 L fermentation medium. The fermentation medium for HA production contained 20 g/L peptone 205, 12.5 g/L phosphates, 10 g/L Pyruvate, 6 g/L Yeast extract, 3 g/L $MgSO_4{\cdot}7H_2O$, 1 g/L *N*-acetylglucosamine, 80 mg/L phosphatidylcholines and 80 g/L glucose (Jagannath and Ramachandran, 2010; Sun et al., 2012a). Fermentation conditions are as follows: pH 7.0–7.5, 37 °C, aeration rate 3 L/min, and agitation speed from 200 rpm to 600 rpm to maintain DO 20–30 % (Liu et al., 2008). Biomass concentration, production of HA, and consumption of glucose were monitored periodically.

Glucose concentration was measured by an SBA-40D biosensor analyzer (Shandong, China). The viscosity of fermentation broth was determined using a rotary viscosimeter (NDJ-8S).

2.4. Semi-continuous fermentation by Streptococcus zooepidemicus

Seed culture was cultured in 1-L shake flasks containing 150 ml sucrose-peptone medium. Semi-continuous of *S. zooepidemicus* was performed in 3–L bioreactors containing 1.5 L fermentation broth. Fermentation conditions were the same as in 2.3. After 8 h of incubation, cell density (OD_{600}) of around 6.0 was reached in the 1st stage bioreactor. Then, 1.05 L (70 %) of broth in 1st stage bioreactor was transferred into 2nd stage bioreactors, and 1.05 L of fresh medium was supplemented into 1st stage bioreactor to begin the next cycle (Fang et al., 2011; Sun et al., 2012b; Li et al., 2021). And after the 1st bioreactor was cultured again for 2 h, the above operation was repeated by transferring 70 % of the fermentation broth into a new 2nd bioreactor, where the

Table 1
Calculation of productivity and yield of HA in batch and semi-continuous fermentation.

	Batch fermentation	Semi-continuous fermentation		
		The 1st stage	The 2nd stage	Overall
Productivity (g/L/h)	$\frac{\Delta C}{\Delta V}$	$\frac{V \cdot C_1}{V_1 \cdot t_1}$	$\frac{C_2 \cdot V_2 - C_1 \cdot V}{t_2 \cdot V_2}$	$\frac{C_2 \cdot V_2}{t_1 \cdot V_1 + t_2 \cdot V_2}$
Yield of HA (g/g)	$\frac{\Delta C}{\Delta S}$	$\frac{C_1}{S_{01} - S_1}$	$\frac{V_2 C_2 - V C_1}{(V_2 - V) S_{02} + V S_1 - V_2 S_2}$	$\frac{V_2 C_2}{V S_{01} + (V_2 - V) S_{02} - V_2 S_2}$

t_1 and t_2: the incubation times of 1st and 2nd stage bioreactors, respectively.
V_1, and V_2: the effective volumes of liquid in 1st and 2nd bioreactors. V represents the volume of culture liquid pumped from 1st to 2nd bioreactor.
C_1 and C_2: HA concentrations at the end of each batch of fermentation in 1st and 2nd bioreactors, respectively.

Table 2
HA production by UV mutagenized *S. zooepidemicus* strains during 4 continuous generations.

Strains	HA titer (g/L)			
	1st	2nd	3rd	4th
WT	0.91 ± 0.07	0.90 ± 0.08	0.91 ± 0.13	0.92 ± 0.06
SZ07	1.41 ± 0.13	1.35 ± 0.16	1.39 ± 0.14	1.40 ± 0.08
SZ11	1.01 ± 0.10	1.01 ± 0.11	0.96 ± 0.12	0.91 ± 0.06
SZ19	1.03 ± 0.19	1.02 ± 0.16	0.98 ± 0.14	0.95 ± 0.17
SZ29	0.99 ± 0.08	1.0 ± 0.14	1.0 ± 0.21	0.99 ± 0.13

same volume of fresh medium was added to the 1st bioreactor. Recombinant hyaluronidase *Sz*HYal (1.5–45 ml) was added after 6 h of each 2nd stage fermentation cycle, and glucose (800 g/L) was added at a flow rate of 5 g/L/h when the glucose concentration was below 20 g/L. Calculations of productivity and yield of HA in batch and semi-continuous fermentation are shown in Table 1. Samples were taken every 2–6 h, and the residual glucose, HA titer, and cell density were measured.

2.5. Analytical methods

After adding equal volume of 0.1 % sodium dodecyl sulfate, samples of culture broth were incubated for 15 min. Then solid impurities were removed by centrifugation for 15 min at 11,000 rpm. Four volumes of 90 % ethanol were added to the supernatant and stirred for 5 min, followed by incubation at around 25 °C for 20 h. The precipitated HA was isolated by centrifugation (12,000 rpm, 25 °C, 20 min), dried at 65 °C for 24 h, and redissolved in 5 g/L NaCl (Wang et al., 2020). The HA concentration was determined by carbazole assay after the redissolved HA solution was appropriately diluted (Bitter and Muir, 1962).

The intrinsic viscosity of the HA solution was measured at 37 °C by Ubbelohde Dilution Capillary system using 5 g/L NaCl as the diluent.

The average Mw of HA was calculated following the Mark–Houwink–Sakurada equation (Eq. A) (Pourzardosht and Rasaee, 2017).

$$[\eta] = 0.0292 \times MW^{0.7848}$$

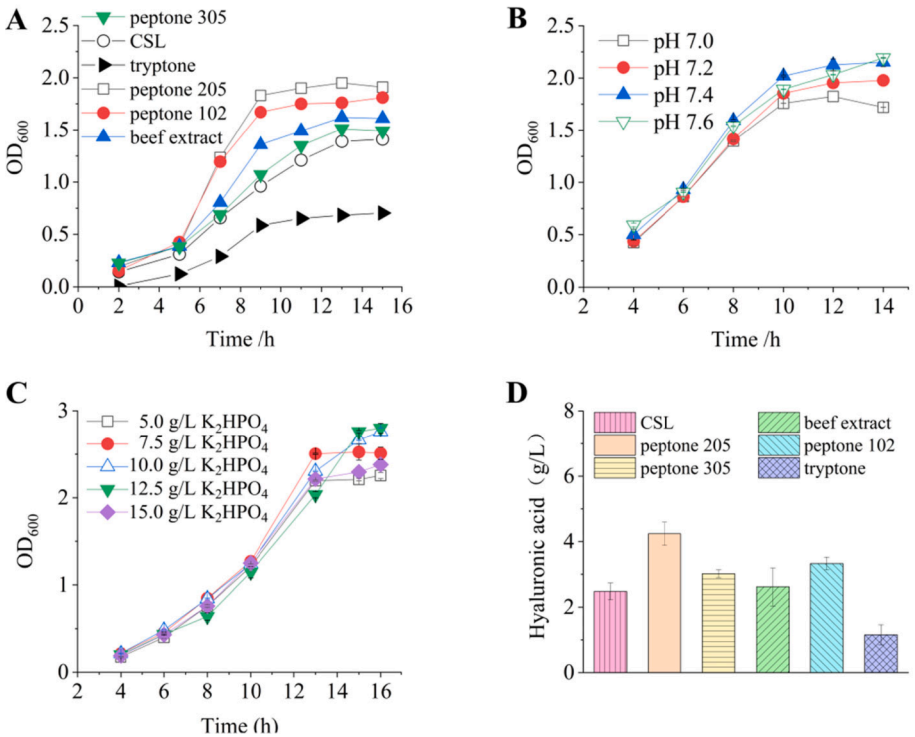

Fig. 1. Optimization of fermentation conditions of *S. zooepidemicus* SZ07. Fermentation was carried out in 500-mL shake flasks containing 100 ml medium. (A) Effects of nitrogen sources, (B) initial pH, and (C) concentrations of K_2HPO_4 on cell growth of SZ07. (D) Effect of nitrogen sources on HA production after 36 h of fermentation.

3. Results and discussion

3.1. UV mutagenesis for enhanced hyaluronic acid production

Various genetic engineering strategies have been developed to improve industrial microorganisms, and UV mutagenesis is still a simple and effective approach (Attia et al., 2021; Wang et al., 2022). Here, UV mutagenesis of *S. zooepidemicus* ATCC 39920 was conducted to generate mutant strains with improved HA production. Different UV irradiation times were attempted, and the lethality of UV light against *S. zooepidemicus* was determined (see supplementary material). The lethality ratio was 78.5 % at 100 s, which was used in further study. After UV irradiation, cells were poured into fresh seed medium and cultured statically in dark for 24 h at 37 °C. Thirty single colonies with apparent white precipitation circles were identified from 100 plates for further screening. HA production of the above 30 mutants were monitored in shake-flasks fermentation (see supplementary material). Among them, SZ07, SZ11, SZ19, and SZ29 exhibited relatively higher HA titers of 1.00–1.41 g/L. The hereditary stability of these 4 strains was further evaluated by subculture for 4 generations (Table 2). Stable HA production of 1.35–1.41 g/L after 36 h of fermentation in shake flasks was observed for SZ07, which was selected for subsequent fermentation in bioreactors. Hyaluronidase production in the exponential phase has been reported to affect HA accumulation by *S. zooepidemicus* (Samadi et al., 2022; Pourzardosht and Rasaee, 2017). Here, gene *hyl* of SZ07 was sequenced, and frameshift mutation caused by T deletion at 201 was observed (see supplementary material).

3.2. Optimization of fermentation conditions

In preliminary fermentation experiments, the cell density (OD_{600}) of SZ07 was lower than 1.0 in shake-flask fermentation. Therefore, the low cell density could be a major limiting factor for HA production by SZ07 (Ozcan et al., 2022). Since glucose has been regarded as an excellent carbon source for *Streptococcus sp*. to produce HA (Im et al., 2009). Therefore, it was used in this study. Phosphates are essential nutrients and buffer salts for microbial cell growth and product synthesis, and are commonly used in the culture of *S. zooepidemicus* (Ozcan et al., 2022; Patil et al., 2011). Then, the initial pH, various nitrogen sources, and phosphate concentration of the medium were optimized to improve HA production and cell growth of SZ07.

Nitrogen sources have significant influences on microbial growth and HA production (Wang et al., 2016). As shown in Fig. 1A and 1D, the highest OD_{600} of 1.91 was achieved at 12 h using peptone 205, leading to the highest HA titer of 4.2 g/L after 36 h of fermentation. At the stationary phase (9–15 h), higher OD_{600} values were accompanied by further increase in HA titer (Fig. 1A and D). As expected, a positive correlation between the HA production and cell growth was observed (Ozcan et al., 2022).

Initial pH of the medium has a great impact on cell growth (Kim et al., 2006). Among different initial pHs tested, higher OD_{600} was achieved with the increasing initial pH from 7.0 to 7.4 (Fig. 1B). Although similar OD_{600} value could be reached at pH 7.6 after 14 h, lower cell density was observed in the earlier fermentation stage of pH 7.6 than that of pH 7.4. Therefore, the initial pH of 7.4 was selected.

The effect of different K_2HPO_4 concentrations on microbial growth was investigated (Fig. 1C). The highest OD_{600} value of 2.84 was reached at 12.5 g/L K_2HPO_4 after 14 h of fermentation.

Based on above results, the optimal fermentation conditions for SZ07 were determined as follows: 20 g/L peptone 205, 12.5 g/L K_2HPO_4, 37 °C and pH 7.4. The highest titer of HA (4.2 g/L) could be obtained after 36 h of fermentation in shake flasks under the optimized conditions (Fig. 1D).

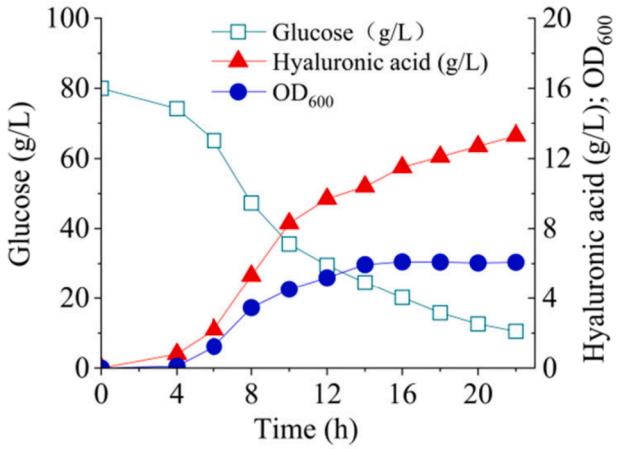

Fig. 2. Batch fermentation for HA production by *S. zooepidemicus* SZ07 in a 3–L bioreactor.

3.3. Batch fermentation for hyaluronic acid production in 3–L bioreactors

In order to further explore the performance of strain SZ07, batch fermentations were performed in 3–L bioreactors. Under initial glucose of 80 g/L, 13.3 g/L HA was achieved with a productivity of 0.60 g/L/h at 37 °C, and a yield of 0.19 g/g glucose (Fig. 2). Notably, high viscosities of over 30 Pa·s was determined after 22 h, which could render poor mass transfer, low dissolved oxygen, and limited HA production.

3.4. Two-stage semi-continuous fermentation process for hyaluronic acid production

HA has been mainly produced by batch and fed-batch fermentation (Qiu et al., 2021; Ucm et al., 2022). And HA titer is often severely hampered by the high viscosity of fermentation broth. In previous reports, a significantly increased dilution ratio could improve the volumetric oxygen transfer coefficient and thus HA production (Saharkhiz and Babaeipour, 2021). Here, a two-stage semi-continuous fermentation process for HA production by SZ07 was developed. First, the dilution rate of 1st stage bioreactor was optimized (Fig. 3A, B). When the fermentation time of 1st stage bioreactor was controlled at 2 h, both HA titer and OD_{600} value were decreased when the dilution rate was increased from 0.20 h^{-1} to 0.45 h^{-1}. The highest HA productivity of 1.63 g/L/h was reached with HA titer of 4.67 g/L at a dilution rate of 0.35 h^{-1}, while the highest HA titer of 4.92 g/L and OD_{600} of 3.96 were obtained at 0.20 h^{-1} (Fig. 3A). When the fermentation time of 1st stage was maintained at 3 h, the highest HA titer of 7.40 g/L was reached at a dilution rate of 0.10 h^{-1}, while the productivity was merely 0.74 g/L/h. At an enhanced dilution rate of 0.25 h^{-1}, the highest productivity of 1.38 g/L/h was attained (Fig. 3B), which is lower than that achieved at 0.35 h^{-1} with 1st stage fermentation time of 2 h (1.63 g/L/h). Considering both HA productivity and titer, the optimum condition of 1st stage bioreactor was determined to be fermentation time of 2 h and dilution rate of 0.35 h^{-1}.

As shown in Fig. 3C, HA production using a two-stage semi-continuous fermentation process was carried out in 3–L bioreactors. During the 6th cycle of 14-h fermentation process (1st stage: 2 h; 2nd stage: 12 h), 14.6 g/L of HA was achieved with a productivity of 1.01 g/L/h and a yield of 0.20 g/g glucose. The viscosity of fermentation broth was determined to be 36 Pa·s, and the average Mw of HA product was calculated to be $9.80 \pm 0.2 \times 10^5$ Da. After the first cycle, HA productivity of 1.60 g/L/h and OD_{600} of 6.20 were achieved in 1st stage bioreactor. In the initial 4 cycles, the final HA concentration and OD_{600} gradually increased in the 2nd stage bioreactors, and the final HA titers of 14.04–14.60 g/L were achieved in the 4th–6th cycles. It is presumed that strain SZ07 was continuously activated and maintained at

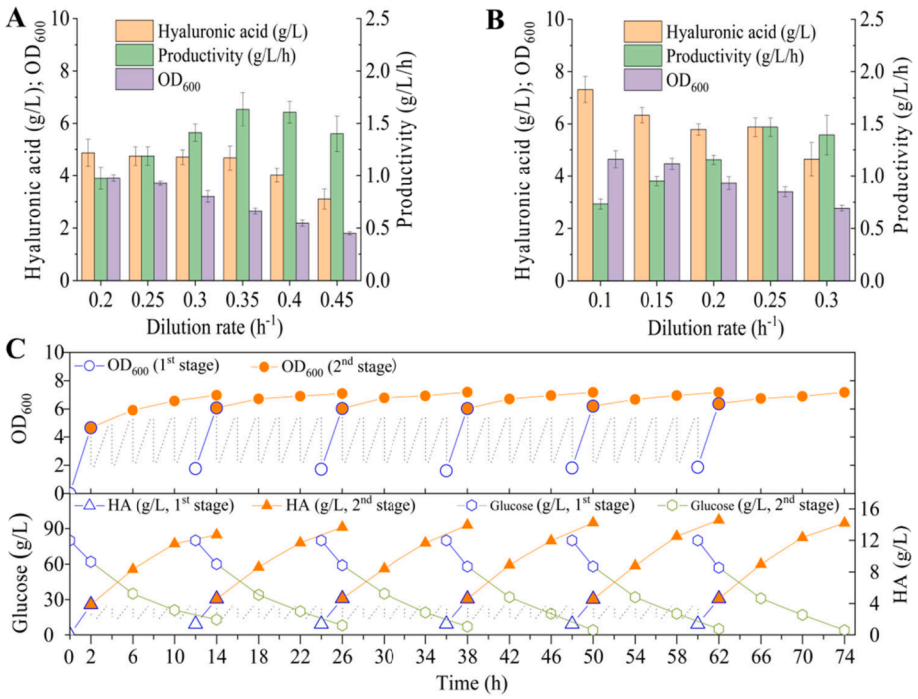

Fig. 3. Two-stage semi-continuous fermentation for HA production by *S. zooepidemicus* SZ07 in 3–L bioreactors. (A) Optimization of dilution rate of 1st stage bioreactor at fermentation time of 2 h and (B) 3 h. (C) Time course of 1st and 2nd stage bioreactors. Dashed lines indicate batches that were not transferred from 1st to 2nd stage bioreactors.

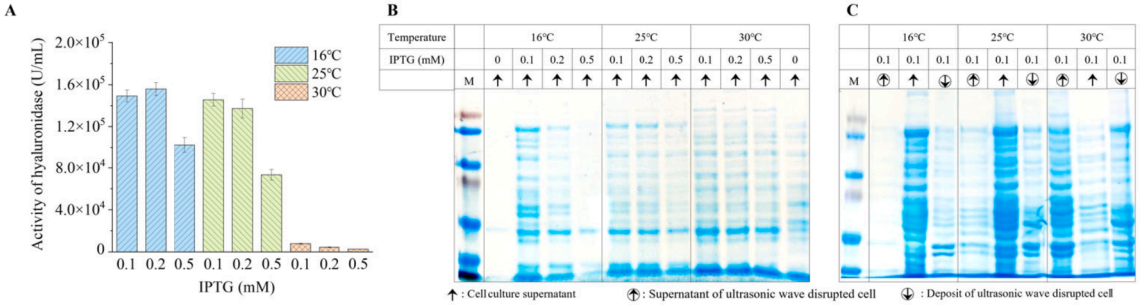

Fig. 4. Effect of different induction conditions on expression of hyaluronidase. (A) 0.1 mM, 0.2 mM, and 0.5 mM IPTG at 16 °C, 25 °C, and 30 °C, respectively. (B) and (C) SDS-PAGE analysis after 48 h of induction.

logarithmic growth phase in the 1st stage bioreactors along with the increasing cycles.

3.5. Addition of hyaluronidase for enhanced hyaluronic acid production

In the later stage of fermentation, the viscosity of broth is getting higher with the accumulation of HA, leading to reduced dissolved oxygen and pH which are often undesirable for HA production (Hiruta et al., 1997; Pourzardosht and Rasaee, 2017). Moreover, cell metabolism is severely limited by the high viscosity of broth (Wei et al., 2019). *Sz*HYal, hyaluronidase from *S. zooepidemicus*, has been reported to hydrolyze mucopolysaccharides into smaller molecules by breaking β-1,4-glycosidic bond of HA, thereby reducing the viscosity of fermentation broth and enhancing HA synthesis and cell growth (Liu et al., 2009). Here, recombinant *E. coli* was constructed for extracellular expression of *Sz*HYal with pelB as signal peptide. The soluble expression of *Sz*HYal gradually decreased at higher temperatures and the ratio of extracellular to intracellular *Sz*HYal decreased with the increasing induction temperature from 16 °C to 30 °C (Fig. 4B and 4C). The highest extracellular hyaluronidase activity of 1.59 × 10^5 U/mL was achieved by induction with

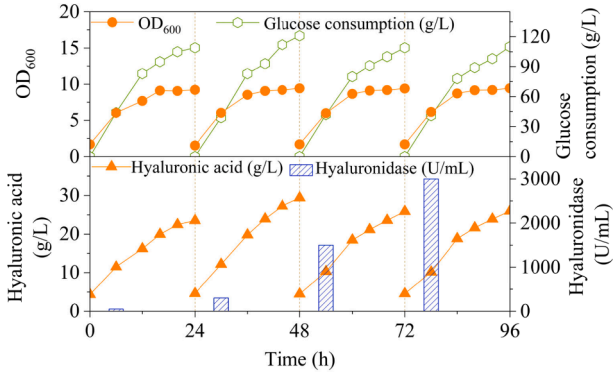

Fig. 5. Time course of 2nd stage bioreactors in two-stage semi-continuous fermentation for HA production by *S. zooepidemicus* SZ07 with *Sz*HYal addition. *Sz*HYal were added into 2nd stage bioreactors at 6 h, and the fermentation time was 24 h for each cycle of 2nd stage.

Table 3
Parameters of different fermentation processes for HA production by *S. zooepidemicus* SZ07.

Parameters	Batch fermentation	Semi-continuous fermentation	Semi-continuous fermentation (50–3000 U/mL *Sz*HYal)
Consumed glucose (g/L)	72 ± 2	72 ± 4	109–121
HA (g/L)	13.3 ± 0.5	13.9 ± 0.7	23.5–29.4
OD_{600}	6.1 ± 0.3	7.2 ± 0.4	9.2–9.4
Yield (%)	19.1 ± 0.002	19.0 ± 0.003	17.6–20.5
HA Productivity (g/L/h)	0.61 ± 0.03	0.96 ± 0.05	0.90–1.13
Viscosity of broth (Pa·s)	32 ± 2	36 ± 9	5.9–24

0.2 mM IPTG at 16 °C after 48 h (Fig. 4A).

To reduce the viscosity and increase the dissolved oxygen of fermentation broth, semi-continuous fermentation with *Sz*HYal addition was carried out. After 6 h of fermentation, *Sz*HYal of 50, 300, 1500, 3000 U/mL were added into the 2nd stage bioreactor in 1st–4th cycles, respectively (Fig. 5). As expected, the OD_{600} and glucose consumption of SZ07 were enhanced, and the HA titer was also significantly increased with the addition of *Sz*HYal (Fig. 5). The fermentation time in the 1st stage bioreactor was still 2 h and in the 2nd stage bioreactors had been extended to 24 h. Compared with semi-continuous fermentation without *Sz*HYal, the total consumption of glucose was increased from 76 g/L (no *Sz*HYal, Fig. 3) to 109, 121, 109, 110 g/L in the 4 cycles, respectively (Fig. 5), and the OD_{600} was increased from 7.2 (no *Sz*HYal, Fig. 3) to 9.2–9.4 (Fig. 5). Importantly, the HA titer was greatly improved from 14.60 g/L (no *Sz*HYal, Fig. 3) to 23.51, 29.38, 25.88, 25.96 g/L in the 4 cycles (Fig. 5), with the highest HA titer of 29.38 g/L achieved at 300 U/mL *Sz*HYal. Fermentation parameters of batch and semi-continuous processes for HA production by *S. zooepidemicus* SZ07 were summarized in Table 3. These results demonstrate that reduced broth viscosity could restore cell metabolism, and promote cell growth as well as HA production.

4. Conclusion

By UV mutagenesis of *S. zooepidemicus* ATCC 39920, a mutant strain SZ07 with enhanced HA production was obtained. Using a newly developed two-stage semi-continuous fermentation process, HA productivity of 1.01 g/L/h and titer of 14.60 g/L were reached at dilution rate of 0.35 h^{-1}, representing 60.3 % and 9.8 % improvement than those in batch fermentation. Recombinant hyaluronidase *Sz*HYal was supplemented to 2nd stage bioreactors to reduce the viscosity of broth, resulting a greatly enhanced HA titer of 29.38 g/L and productivity of 1.13 g/L/h. This study provides practical guidance for developing efficient fermentation process for producing HA and related polysaccharides.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Compliance with ethical standards

Ethical statement: This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.128896.

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