Applied Microbiology and Biotechnology (2019) 103:4363–4375
https://doi.org/10.1007/s00253-019-09769-0

BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Production of controlled molecular weight hyaluronic acid by glucostat strategy using recombinant *Lactococcus lactis* cultures

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Received: 4 January 2019 / Revised: 11 March 2019 / Accepted: 12 March 2019 / Published online: 9 April 2019

Abstract

Hyaluronic acid (HA) is a biopolymer with wide biomedical and cosmetic applications, wherein the molecular weight of HA (MW_{HA}) is an important quality parameter that determines its suitability for the targeted application. To produce HA with desired molecular weight, it is important to identify parameters that offer tunability and control of MW_{HA} at a desired value during fermentation. In this work, two tunable parameters, viz. glucose concentration and combination of HA biosynthetic genes expressed, were used to produce HA of different molecular weights. Three recombinant strains of *Lactococcus lactis* were constructed, using a combination of the *has*-operon genes from *Streptococcus zooepidemicus* (*hasA*, *hasB*, *hasE*) and the α-phosphoglucomutase gene (*pgmA*) from *L. lactis*. Batch fermentations of these recombinant strains at different initial glucose concentrations enabled production of HA with different molecular weights. Co-expression of *hasABE* was observed to be particularly effective in improving the MW_{HA}. It was observed during batch fermentations of all these recombinant *L. lactis* cultures that the MW_{HA} decreases steadily during the later part of the fermentation and the final value is 19–43% lower than the peak MW_{HA} produced. Analysis of the fermentation data showed that the decrease in MW_{HA} correlated strongly with the decrease in specific productivity of the culture. To overcome this decrease in MW_{HA}, a glucostat strategy was successfully devised which could maintain a high value of specific productivity throughout the glucostat phase and result in constant-MW HA production. Glucostat processes were designed with the three recombinant *L. lactis* strains at two different glucose concentrations to produce constant molecular weight HA ranging from 0.4 to 1.4 MDa. This is the first report of its kind in literature that demonstrates production of controlled MW HA over a wide range by using a combination of tunable parameters and suitable process control strategies.

Keywords Hyaluronic acid · *Lactococcus lactis* · Molecular weight variation · Glucostat · Constant molecular weight HA

Introduction

Hyaluronic acid (HA) is a biopolymer with wide applications in the fields of medicine and cosmetics. Molecular weight of HA (MW_{HA}) is an important quality parameter that determines its suitability for various end applications (Table S1, Supplementary data). Therefore, strategies to produce HA with the desired molecular weight will be beneficial to industry (Marcellin et al. 2014). Developing such strategies requires identifying parameters that allow production of HA over a wide range of molecular weights and control of molecular weight at a desired value. There is very limited work on identifying tunable parameters which affect MW_{HA} (Jing and DeAngelis 2004; Sheng et al. 2009) and on controlling MW_{HA} at a desired value during fermentation (Jin et al. 2016).

Literature reports show many genetic and process parameters which affect MW_{HA}. Genetic parameters include the combination of heterologous *has* genes (in the HA biosynthetic pathway) being overexpressed (Yu and Stephanopoulos 2008; Chauhan et al. 2014); whether the *has* genes were plasmid-encoded or chromosomally integrated (Hmar et al. 2014); gene-knockout in competing pathways which enhanced

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00253-019-09769-0) contains supplementary material, which is available to authorized users.

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co-factor availability for HA-precursor synthesis (Kaur and Jayaraman 2016); downregulation of the glycolytic pathway and regulated production of hyaluronidase (Jin et al. 2016) (Table S2, Supplementary data). Process parameters such as pH, agitation rate, aeration rate, temperature, initial glucose concentration, C/N ratio (Kim et al. 1996; Armstrong and Johns 1997; Duan et al. 2008; Jagannath and Ramachandran 2010; Lai et al. 2011) have also been shown to affect MW_{HA} (Table S3, Supplementary data). However, the main objective in most literature reports is to optimize the parameters in order to obtain the maximum possible MW_{HA}. A few literature reports have demonstrated modulation of MW_{HA} using tunable parameters. Sheng et al. (2009) varied the *hasA/hasB* mRNA ratio, which resulted in HA molecular weight variation only in a narrow range of 0.6–0.9 MDa. Jin et al. (2016) used a combination of genetic strategies to produce HA in the molecular weight range of 2.2 kDa to 1.42 MDa in engineered *Bacillus subtilis* strains. They initially produced HA in the MW range of 1.4–1.83 MDa by co-expression of multiple genes in the HA pathway and downregulation of the *pfk* gene in the glycolytic pathway. Then, they co-expressed regulated amounts of engineered leech hyaluronidase to produce HA over a wide molecular weight range. Jing and DeAngelis (2004) varied the precursor to acceptor oligosaccharide ratio during in vitro HA polymerization to obtain monodisperse HA with molecular weight in a wide range of 16 kDa to 2 MDa. However, this strategy offers poor scalability owing to its expensive nature.

Another drawback with most literature reports on HA production is that these studies have only reported the MW_{HA} obtained at the end of a batch fermentation process. There are hardly any reports on variation of MW_{HA} during the course of the batch fermentation. Only four reports on HA production using *S. zooepidemicus* provide HA molecular weight trend as a part of the fermentation profile. Armstrong and Johns (1997) and Im et al. (2009) observed an initial increase in MW_{HA} followed by a constant molecular weight trend for the HA produced. Two other reports have shown that, after an initial increase, the MW_{HA} decreased by 30–60% during the course of the fermentation (Pires and Santana 2010; Jagannath and Ramachandran 2010). A recent study using four recombinant *B. subtilis* strains showed a 11–31% decrease in MW_{HA} during flask cultivation (Westbrook et al. 2018). None of the above studies investigated the reasons for the decrease in MW_{HA} or provided any strategies to control MW_{HA} during the fermentation.

Similar to the above studies, in this work, we have shown a decrease in MW_{HA} produced by recombinant *Lactococcus lactis* strains during the course of batch fermentations. We have analyzed the process parameters and correlated them with variation in MW_{HA} and devised a process strategy for controlling MW_{HA} during the fermentation process. The molecular weight control strategy developed was employed in fermentations involving different sets of genetic and process parameters which affect molecular weight, thus allowing production of constant molecular weight HA over a wide range of values.

Materials and methods

Chemicals and kits

M17 media components, glucose, and cetyltrimethyl ammonium bromide (CTAB) were purchased from HiMedia Laboratories (India). Isopropyl alcohol, sulfuric acid, and sodium nitrate were purchased from Merck (USA). Hyaluronan standards with M_v-0.1, 0.6, and 1.59 MDa (measured using viscometry) were purchased from Lifecore Biomedical (USA).

The GenElute Bacterial Genomic Kit, chloramphenicol, and nisin were obtained from Sigma-Aldrich (USA). QIAprep Spin Miniprep kit for plasmid isolation, QIAquick™ Gel Extraction kit and the QIAquick™ PCR purification kit were purchased from Qiagen (Germany). The enzymes *Nco*I, *Kpn*I, *Pml*I, Phusion™ DNA Polymerase, and T4 DNA ligase were bought from New England Biolabs (USA).

Construction of *L. lactis* clones overexpressing *hasA*, *hasB*, *hasE*, and *pgmA* genes

Bacterial strains and plasmids

Streptococcus equi subsp. *zooepidemicus* ATCC 35246 was obtained from American Type Culture Collection (USA). The plasmid pNZ8148 and *Lactococcus lactis* NZ9000 were obtained from NIZO (Netherlands). *E. coli* MC1061 was a kind gift from Dr. Devinder Sehgal, National Institute of Immunology, New Delhi.

Construction of recombinant *Lactococcus lactis*

The gene sequences of *hasA*, *hasB*, and *hasE* were obtained from the partial sequence of *Streptococcus equi* subsp. *zooepidemicus has* operon, with GenBank accession ID-AF347022.1. The base positions of *has* genes in this partial sequence were *hasA*-512 to 1784; *hasB*-2023 to 3242; *hasE*-590 to 7268. Similarly, *pgmA* gene sequence was obtained from the complete genome sequence of *Lactococcus lactis* subsp. *cremoris* NZ9000 with GenBank accession ID-CP002094.1. The base position of *pgmA* gene in this complete genome sequence was 540,061 to 540,819.

Nisin inducible plasmid, pNZ8148, was used to construct recombinant plasmids pGJP2 (containing *hasA* and *hasB* genes), pGJP5 (containing *hasA*, *hasB* and *hasE* genes) and pGJP6 (containing *hasA*, *hasB* and *pgmA* genes). The pGJP2 plasmid was constructed first, followed by cloning of *hasE*

and *pgmA* genes into pGJP2 to obtain pGJP5 and pGJP6 constructs, respectively. The step-wise cloning strategy used for constructing the recombinant plasmids pGJP2, pGJP5 and pGJP6 is shown schematically in Fig. S1a and S1b, Supplementary data. The recombinant clones after transformation of pGJP2, pGJP5 and pGJP6 into *L. lactis* NZ9000 were assigned as *L. lactis* GJP2, GJP5 and GJP6, respectively.

The genomic DNA of *S. equi* subsp. *zooepidemicus* ATCC 35246 and *L. lactis* NZ9000 were isolated using GenElute™ Bacterial Genomic DNA Kit. Using the Phusion™ High-Fidelity DNA Polymerase, *hasE* gene and a single DNA fragment comprising *hasA* and *hasB* genes were amplified from *S. zooepidemicus* genomic DNA. The *pgmA* gene was amplified from *L. Lactis* NZ9000 genomic DNA. The primers used for amplification of different gene fragments and the associated restriction sites are shown in Table S4 (Supplementary data). The PCR conditions for amplification of the gene fragments were as follows: after an initial denaturation at 98 °C for 2 min, subsequent denaturation was carried out at 98 °C for 30 s. Annealing was carried out at 52 °C for *hasAB* gene fragment and at 47 °C for *hasE* and *pgmA* genes, each for 45 s. The extension step was carried out at 72 °C for varied time periods for different fragments as follows: *hasAB* for 90 s, *hasE* for 30 s and *pgmA* for 30 s. The total number of cycles (starting from second denaturation to extension) was 35 cycles for all amplifications. The final extension for all gene combinations was at 72 °C for 5 min. The amplicons were then purified using QIA quick™ PCR purification kit.

The vector pNZ8148 and the gene fragment were double digested with respective restriction enzymes (Fig. S1c, Supplementary data) for 3 h at 37 °C and purified using QIAquick™ Gel Extraction kit and ligated with T4 DNA ligase at 16 °C for 16 h. The ligated products were transformed into ultra-competent *E. coli* MC1061 and plated on Luria-Bertani agar plates containing chloramphenicol (10 μg/ml) and streptomycin (10 μg/ml). The positive recombinants screened using restriction digestion and PCR were confirmed by DNA sequencing. The positive recombinant plasmids were then transformed into electrocompetent *L. lactis* by electroporation.

Fermentation experiments

All bioreactor experiments were carried out in duplicate in an unaerated 2.4 L bioengineering (Switzerland) bioreactor at 30 °C, 200 rpm, and pH 7. Modified M17 media (1.2 L) with the following composition was autoclaved in situ: yeast extract-5 g/l, brain heart infusion-5 g/l, $MgSO_4 \cdot 7H_2O$-0.5 g/l, ascorbic acid-0.5 g/l, KH_2PO_4-0.5 g/l, and K_2HPO_4-1.5 g/l. Autoclaved glucose added with chloramphenicol (10 μg/ml) was aseptically transferred into the reactor. Inoculum was prepared from glycerol stocks stored at − 80 °C by sub-culturing twice in flasks and was aseptically introduced into the fermenter to a final concentration of 8%.

For glucostat experiments, glucose concentration in the bioreactor was maintained constant by using pH feedback strategy (Papagianni et al. 2007). The glucose consumed for lactic acid production can be monitored from the amount of base added by the pH controller and a correlation can be obtained between the two. It was found that for every gram of glucose consumed, 0.578 g of KOH was pumped in (Fig. S2, Supplementary data). Glucose feed and KOH were both connected to the base pump of the fermenter. Glucose and KOH solutions were prepared such that, with every instance of addition of base, the corresponding amount of glucose consumed in the reactor will be replenished by the base pump. For all bioreactor experiments, samples were drawn every 2 h and stored at − 20 °C until they were analyzed for concentrations of glucose, biomass, HA, lactate, and HA molecular weight.

Analytical techniques

Determination of biomass concentration

Biomass concentration was determined from absorbance measured at 600 nm. Fermentation broth was treated with equal volume of 0.1% SDS for 10 min at room temperature and centrifuged at 9500×*g* for 10 min. The pellet was re-suspended in 0.9% NaCl and absorbance was measured at 600 nm using UV-visible spectrophotometer (Jasco, US).

Determination of glucose and lactate concentration

Glucose and lactate concentration in the broth were measured using ion exchange chromatography. Phenomenex Rezex R-OA column (320 mm × 4.5 mm) along with Phenomenex Rezex R-OA guard column fitted to Shimadzu HPLC-PDA-RID detector system was used for the analysis. Isocratic elution using 5 mM sulfuric acid with a flow rate of 0.6 ml/min was carried out. Lactate concentration was measured from absorbance at 210 nm and glucose concentration was measured using RID.

Determination of HA concentration

HA concentration was measured using CTAB method (Oueslati et al. 2014). Fermentation broth was treated with equal volume of 0.1% SDS for 10 min at room temperature and centrifuged at 9500×*g* for 10 min. The supernatant obtained was used for the CTAB assay.

Determination of HA molecular weight

MW_{HA} was measured using size exclusion chromatography (SEC). Calibration of SEC and molecular weight estimation of

HA in fermentation samples using SEC were carried out according to Shanmuga Doss et al. (2017).

Statistical analysis

The statistical analysis was performed using *t* test available in Minitab 16 with 5% level of significance. All fermentation experiments and analyses were performed at least in duplicate, and the results were expressed as a mean ± standard deviation. Statistical significance was represented by *p* values $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Results

The two objectives of this work were (i) to devise process strategies to control variation in MW_{HA} during fermentation and (ii) to produce constant-MW HA over a wide range of molecular weights. Batch experiments were carried out using the three recombinant *L. lactis* strains to validate production of HA with different molecular weights. During batch fermentation with all three recombinant strains, the MW_{HA} showed a decreasing trend after a small initial phase of increase. From the analysis of bioprocess parameters during the batch fermentation experiments, the decrease in MW_{HA} was inferred to be due to the drastic reduction of glucose flux towards HA biosynthetic pathway. This observation correlated with the decreasing glucose concentration towards the later part of the batch fermentation. Therefore, a glucostat strategy was developed and tested for production of constant-MW HA during fermentation. The simultaneous manipulation of process parameter (glucose concentration) and genetic parameter (combination of HA biosynthetic genes overexpressed) in a glucostat enabled production of constant-MW HA over a 0.4–1.4 MDa range.

Construction of recombinant *L. lactis* strains

Three recombinant *L. lactis* clones, overexpressing different combinations of HA biosynthetic pathway genes, were constructed using the nisin inducible pNZ8148 expression system. The genes *hasA*, *hasB* and *hasE* (Fig. 1) were amplified from the *has*-operon in the genomic DNA of the natural HA producer *S. zooepidemicus* while the gene coding for α-phosphoglucomutase (*pgmA*) was obtained from *L. lactis*. The recombinant plasmids pGJP2 (containing *hasAB* genes), pGJP5 (containing *hasABE* genes) and pGJP6 (containing *hasAB* and *pgmA* genes) were transformed into *L. lactis* NZ9000. The construction of the recombinant strains is detailed in the "Materials and methods" section.

While *L. lactis* does not have the hyaluronan synthase (*hasA*) gene, it has the homolog (*ugd*) of the *hasB* gene, coding for UDP-glucose dehydrogenase. Since *ugd* is tightly transcriptionally regulated (Prasad et al. 2012), overexpression of both *hasA* and *hasB* is required for substantial production of HA by recombinant *L. lactis* cultures. Earlier studies with recombinant *L. lactis* strains have shown that overexpression of a third HA pathway gene (*hasC* or *hasD*), in addition to *hasA* and *hasB*, results in enhancement of HA production in recombinant *L. lactis* cultures (Prasad et al. 2010; Prasad et al. 2012; Chauhan et al. 2014). In this study, we chose *hasE* and *pgmA* as the third gene, in addition to *hasA* and *hasB*, for overexpression in the recombinant *L. lactis* strains GJP5 and GJP6, respectively. As shown in Fig. 1, *hasE* and *pgmA* express the nodal enzymes from glucose-6-phosphate in the HA biosynthetic pathway. Hence, by selective overexpression of *hasE* and *pgmA*, the carbon flux distribution could be varied in the two parallel pathways leading to the formation of the two precursors of HA, viz., UDP-glucuronic acid and UDP-N-acetyl glucosamine. As the intracellular ratio of the two precursors (and their pathway fluxes) has been shown to have a significant effect on MW_{HA} (Badle et al. 2014), we expected the clones overexpressing *hasE* and *pgmA* genes along with *hasAB* to produce HA of different molecular weights. While overexpression of *pgmA* gene in *S. zooepidemicus* was shown earlier to not affect HA yield and MW_{HA} (Chen et al. 2014), overexpression of *hasE* has been shown to enhance MW_{HA} in *S. zooepidemicus* (Chen et al. 2009).

Effect of co-expression of *hasE* and *pgmA* genes on HA production and molecular weight

Batch fermentation experiments were carried out using the three recombinant *L. lactis* strains at 10 g/l and 30 g/l initial glucose concentration. For sake of clarity, Fig. 2 shows only the profiles of MW_{HA} along with glucose and HA concentration. Complete batch fermentation profiles including biomass and lactate concentration are shown in Fig. S3 (Supplementary data). In accordance with previous literature reports (Jagannath and Ramachandran 2010; Chauhan et al. 2014), a higher initial glucose concentration gave rise to higher HA concentration and MW_{HA} at the end of the batch fermentations for all the three recombinant strains (Fig. 3).

The effect of overexpression of a third gene (*hasE*/*pgmA*) in addition to *hasAB*, on HA concentration and MW_{HA}, is illustrated in Fig. 3. Compared to the *L. lactis* GJP2 strain (expressing *hasAB*), overexpression of *hasABE* in *L. lactis* GJP5 resulted in 120% and 56% improvement in HA concentration for the batch fermentations using 10 g/l and 30 g/l initial glucose concentrations, respectively (Fig. 3a). Similarly, additional overexpression of *pgmA* resulted in 150% and 47% increase in HA concentration under the respective conditions. This implies that overexpression of the third gene improved the carbon flux

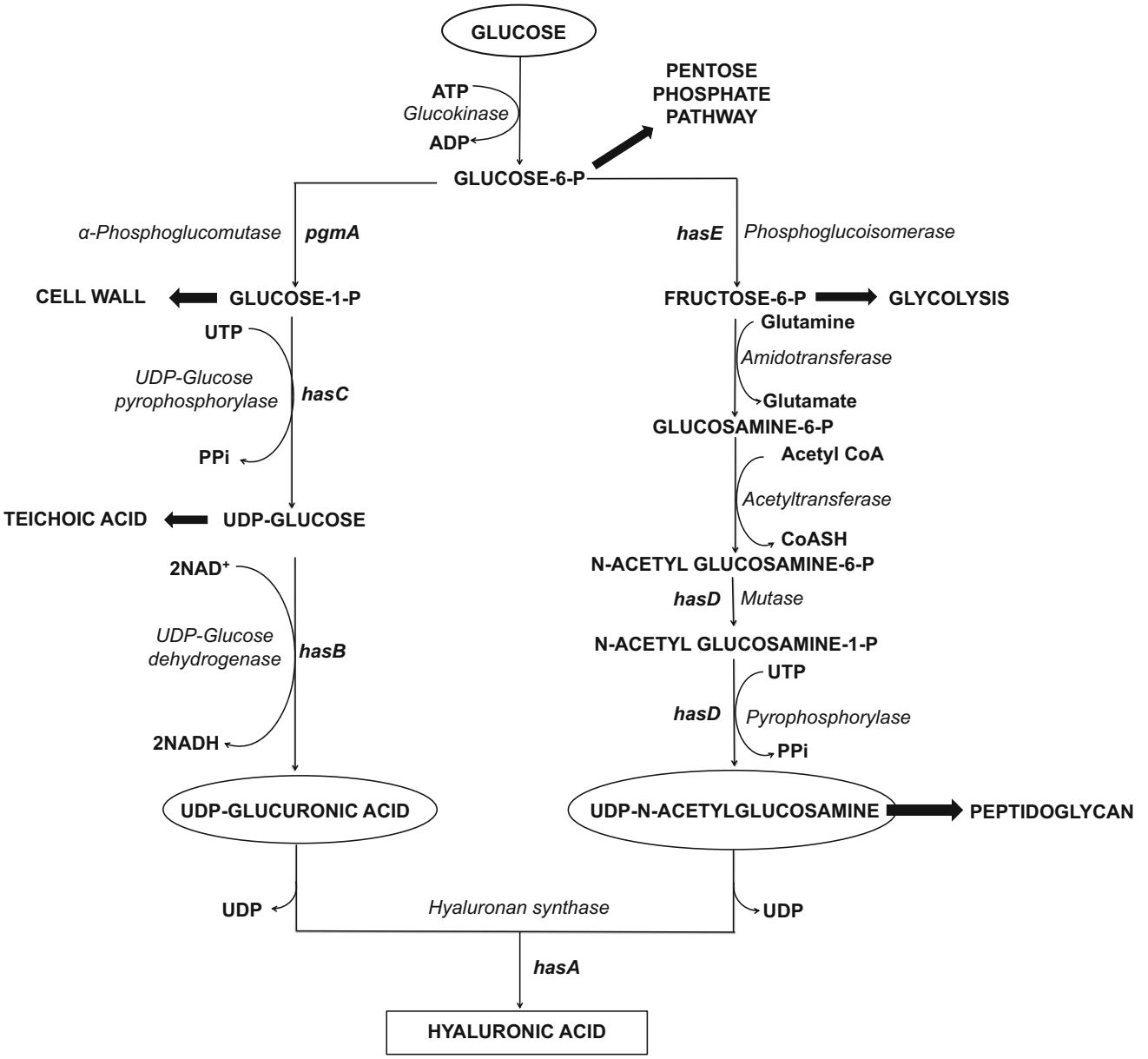

Fig. 1 HA Biosynthetic pathway in *Streptococcus zooepidemicus*

towards the respective HA-precursor, thus improving HA concentration and yield for *L. lactis* GJP5 and GJP6 cultures.

Overexpression of *hasE* gene resulted in significantly higher MW_{HA} towards the end of the batch fermentation (end-MW_{HA}) in comparison to *pgmA* overexpression (Fig. 3b). Compared to *L. lactis* GJP2, *L. lactis* GJP5 cultures produced 84–95% higher end-MW_{HA}, while *L. lactis* GJP6 produced only 21–51% higher end-MW_{HA} during batch fermentations using 10 and 30 g/l initial glucose concentrations. Thus, co-expression of *pgmA* and *hasE* (along with *hasAB*) exhibited an enhancement in MW_{HA}, with *hasE* co-expression showing a more pronounced effect. This increase in MW_{HA} upon coexpression of a third gene along with *hasAB* is in contrast to the results reported by Prasad et al. (2010) and Chauhan et al. (2014), wherein they observed no enhancement of MW_{HA} upon the co-expression of *hasABC* or *hasABD* in *L. lactis* strains.

Molecular weight variation of HA during batch fermentation

The HA molecular weight profile for all the six batch fermentations shows that, after a brief initial increase, the MW_{HA} decreases steadily from its peak value until end of the fermentation (Fig. 2). Across the three recombinant strains, MW_{HA} showed 26–35% decrease and 19–43% decrease during the 10 g/l and 30 g/l batch fermentations, respectively (Fig. 3b). Since MW_{HA} measured is the average molecular weight of all HA chains present in the broth, a decrease in the average

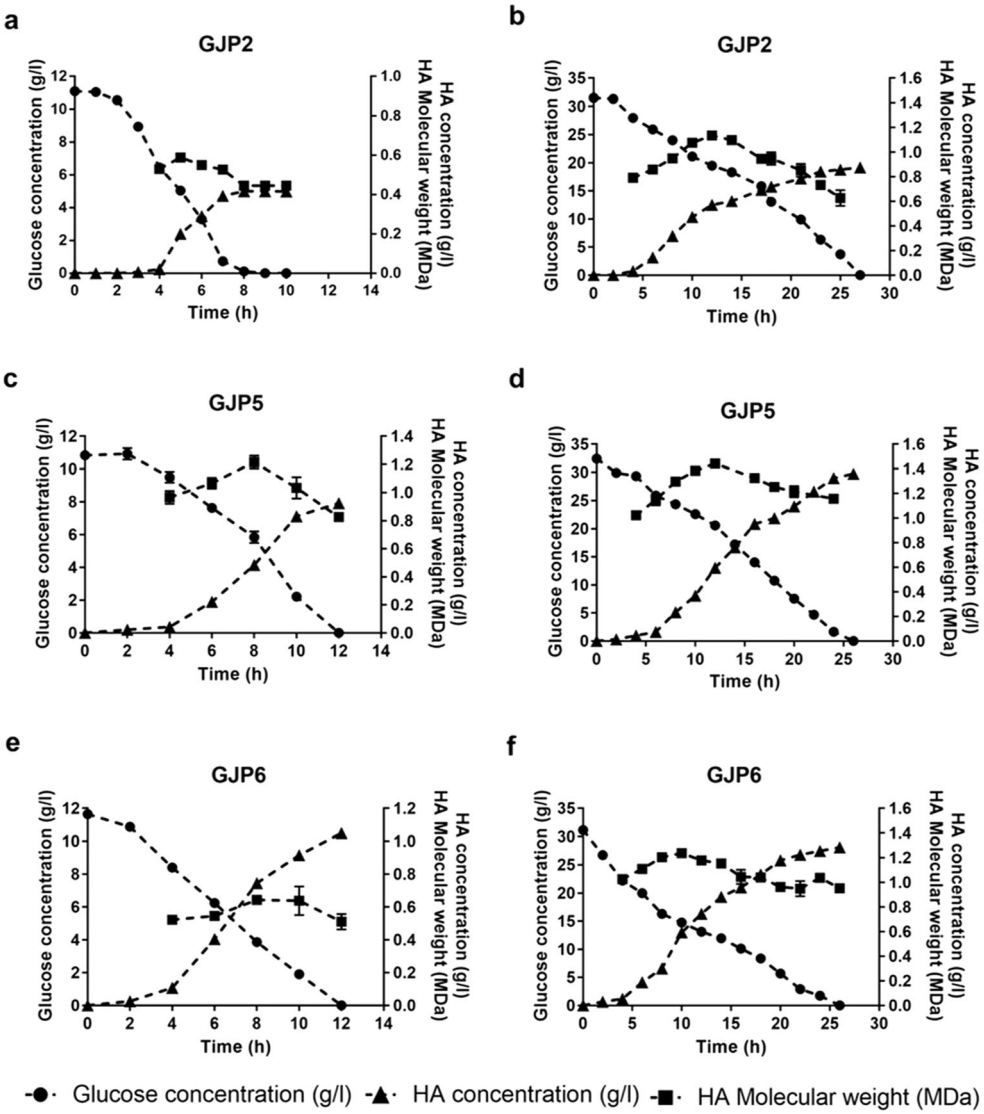

Fig. 2 Batch fermentation profiles of *L. lactis* GJP2, *L. lactis* GJP5 and *L. lactis* GJP6 carried out using initial glucose concentration of **a**, **c**, **e**10 g/l and **b**, **d**, **f** 30 g/l

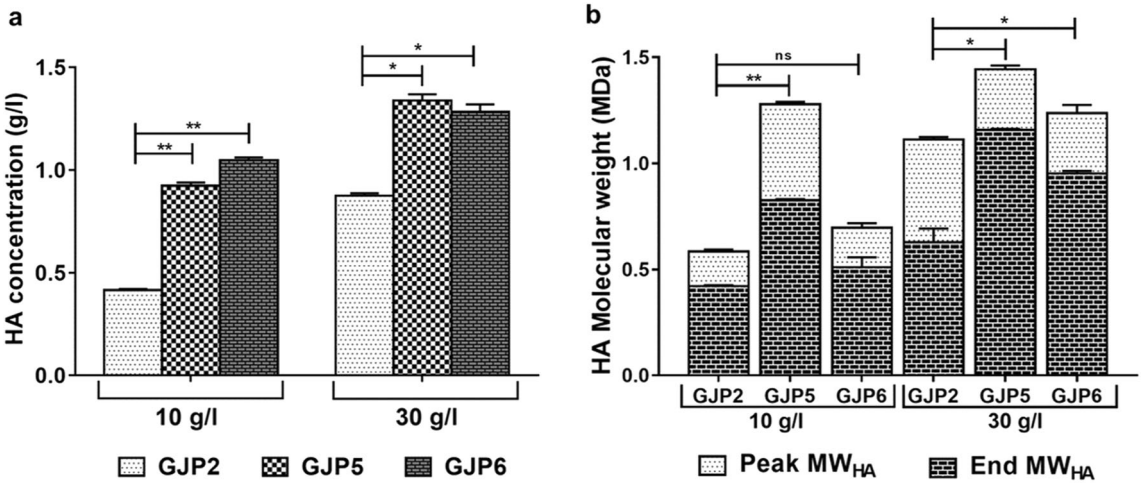

Fig. 3 Comparison of **a** HA concentration, **b** peak and end HA molecular weight obtained from batch fermentation of *L. lactis* GJP2, *L. lactis* GJP5, and *L. lactis* GJP6 carried out using 10 and 30 g/l initial glucose concentration

molecular weight would mean introduction of shorter chains into the broth with progress of fermentation. Shorter chains can be introduced into the broth either by the cell or by degradation of existing HA chains due to shear or hyaluronidase action inside the reactor. Since *L. lactis* is hyaluronidase negative, a control experiment was carried out to check shear degradation of HA inside the reactor. In a batch experiment with un-induced recombinant *L. lactis* culture, 1 g of HA was spiked into the fermenter a few hours after inoculation. Molecular weight profiling revealed that MW_{HA} remained constant throughout the experiment (Fig. S4, Supplementary data), thus ruling out shear degradation during HA production. From Fig. 2, it can be observed that decrease in MW_{HA} is accompanied by increase in HA accumulation during all six cultivations. Hence, the decreasing trend of MW_{HA} might be due to production of shorter HA chains by the cell towards the later part of the batch fermentations.

However, further analysis was needed to explain the decreasing trend of MW_{HA}. We focused on the fermentation parameters such as specific growth rate (μ), specific glucose uptake rate (q_s), specific production rate of HA (q_p) and the various yield coefficients based on glucose uptake, viz. biomass ($Y_{X/S}$), HA ($Y_{HA/S}$), and lactate ($Y_{LA/S}$) yield coefficients (Table 1). Furthermore, the batch fermentation process was divided into two phases, viz. phase of increasing MW_{HA} (phase 1) and phase of decreasing MW_{HA} (phase 2) and the averaged values of the bioprocess parameters were calculated independently for the two phases. One of the critical parameters is the specific production rate of HA (q_p), which is determined by specific glucose uptake rate (q_s) and the proportion of glucose fluxed into HA formation, measured by $Y_{HA/S}$ (C-mol/C-mol). From Table 1, q_s and $Y_{HA/S}$ were observed to decrease with transition from phase 1 to phase 2 for the 10 g/l as well as 30 g/l glucose concentration experiments. Consequently, the q_p values were much lower in phase 2 as compared to phase 1 (Fig. 4a, b) and might reflect lower precursor availability for HA formation. This reduction in q_p and the probable decrease in precursor availability could be the reason for formation of shorter chains and decreasing MW_{HA} during phase 2. The values of $Y_{X/S}$ were consistently lower while that of $Y_{L/S}$ were consistently higher for phase 2 in comparison to phase 1 (Table 1). Papagianni et al. (2007) observed in batch cultures of *L. lactis*, carried out at 10 g/l initial glucose concentration, the specific lactate production rate increased sharply when the glucose concentration dropped to ~5–6 g/l. We observed similar trends in some of the 10 g/l batch fermentations (Fig. S3, Supplementary data). The decrease in q_s, owing to decreasing glucose concentration in the batch fermentation, leads to a redistribution of carbon flux in favor of a higher lactate yield at the expense of biomass and product yield. Therefore, to prevent decrease in MW_{HA}, it is important

Table 1 Comparison of specific glucose uptake rate and yield coefficients of HA, biomass, and lactate based on glucose uptake, between phase of increasing MW_{HA} and phase of decreasing MW_{HA} of batch fermentations of *L. lactis* GJP2, *L. lactis* GJP5, and *L. lactis* GJP6 carried out using 10 and 30 g/l initial glucose concentration

Initial glucose concentration (g/l)	Strain	Phase of increasing MW_{HA}				Phase of decreasing MW_{HA}			
		q_s g/(g.h)	$Y_{HA/S}$ C-mol/C-mol	$Y_{X/S}$ C-mol/C-mol	$Y_{LA/S}$ C-mol/C-mol	q_s g/(g.h)	$Y_{HA/S}$ C-mol/C-mol	$Y_{X/S}$ C-mol/C-mol	$Y_{LA/S}$ C-mol/C-mol
10	GJP2	1.27	0.142	0.133	0.763	0.87	0.054	0.105	0.874
	GJP5	0.96	0.134	0.190	0.686	0.86	0.084	0.120	0.820
	GJP6	0.88	0.156	0.175	0.678	0.59	0.087	0.129	0.870
30	GJP2	0.65	0.07	0.129	0.813	0.52	0.017	0.087	0.955
	GJP5	0.83	0.069	0.143	0.8	0.56	0.043	0.1	0.875
	GJP6	0.74	0.080	0.112	0.784	0.44	0.052	0.071	0.885

Standard deviation in q_s and yield coefficients was < 5% of the corresponding values

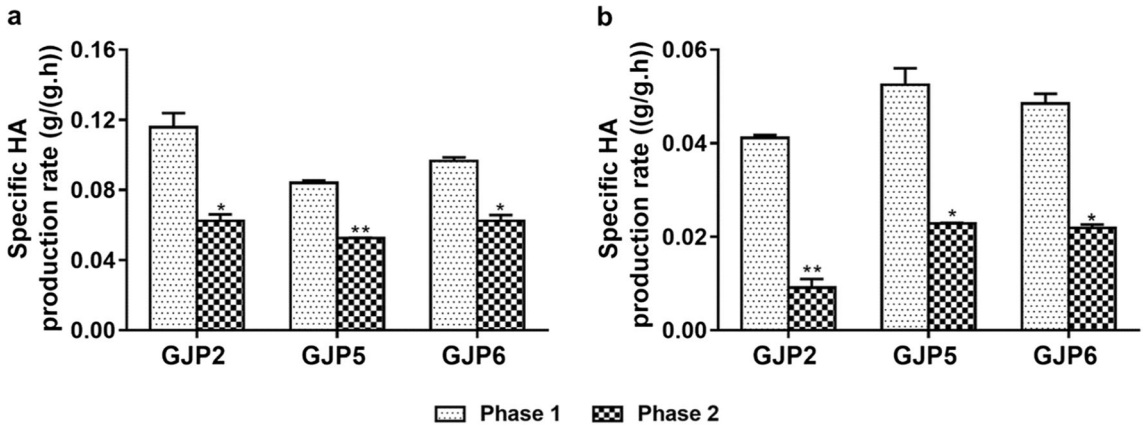

Fig. 4 Comparison of specific HA production rate between phase 1 and phase 2 of batch fermentation experiments carried out using *L. lactis* GJP2, *L. lactis* GJP5, and *L. lactis* GJP6 at an initial glucose concentration of **a** 10 g/l and **b** 30 g/l

to develop strategies to prevent drastic decreases in q_s, $Y_{HA/S}$, and q_p. To sustain high values of these parameters during fermentation, we attempted a glucostat strategy as explained in the next section.

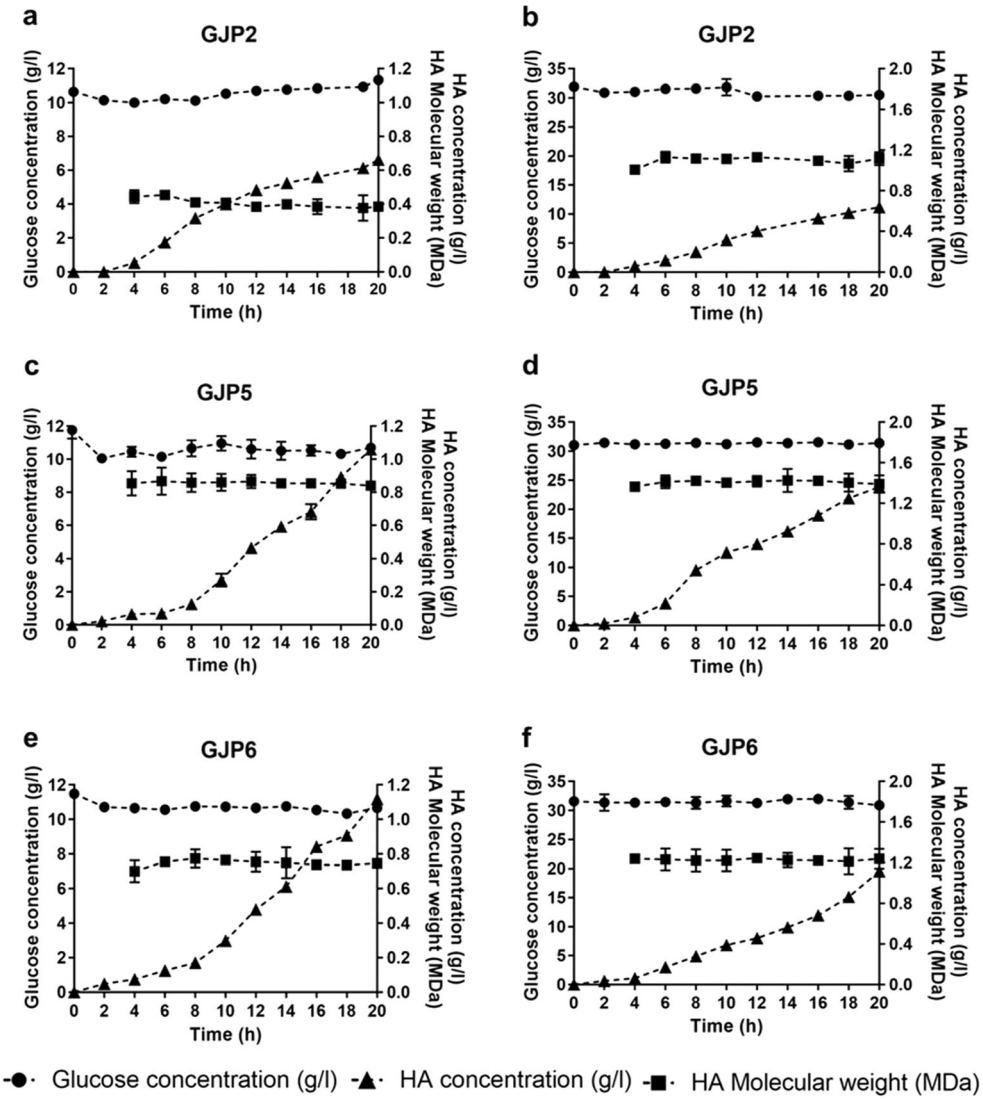

Fig. 5 Glucostat fermentation profiles of *L. lactis* GJP2, *L. lactis* GJP5, and *L. lactis* GJP6 carried out at glucose concentration of **a**, **c**, **e** 10 g/l and **b**, **d**, **f** 30 g/l

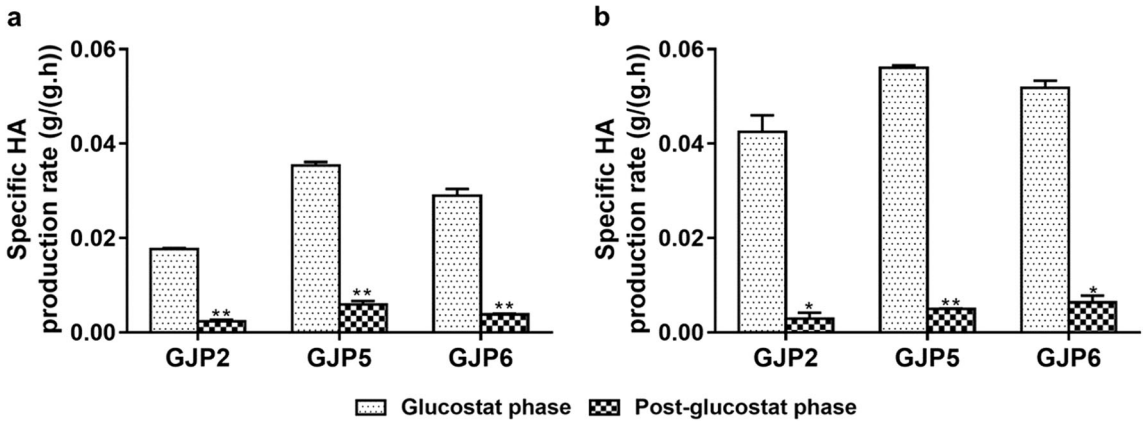

Fig. 6 Comparison of specific HA production rate between glucostat phase and post-glucostat phase of glucostat fermentation experiments carried out using *L. lactis* GJP2, *L. lactis* GJP5, and *L. lactis* GJP6 at a glucose concentration of **a** 10 g/l and **b** 30 g/l

Glucostat strategy for control of MW_{HA} during fermentation

We designed a glucostat process as a possible strategy to prevent the decrease in MW_{HA} which was observed in batch fermentations. Glucostat can be described as a fermentation process in which glucose concentration is maintained constant at a desired value by continuous feeding of glucose. Figure 5 shows the glucose, HA and MW_{HA} profiles for the 10 g/l and 30 g/l glucostat experiments using the three recombinant *L. lactis* strains. Detailed profiles can be found in Fig. S5 (Supplementary data). Figure 5 shows that glucostat fermentation resulted in constant-MW HA production. By carrying out the glucostat fermentation at different glucose concentrations and using the three recombinant *L. lactis* clones overexpressing different combinations of HA biosynthetic genes, constant-MW HA production in a range of 0.4–1.4 MDa was achieved (Fig. S6, Supplementary data).

The continuous production of HA during the glucostat shows that, on the average, HA chains of similar length were produced during this period. This observation is in contrast to the decreasing trend of MW_{HA} during batch fermentations and clearly shows the power of glucose concentration as a control parameter. A constant glucose concentration in the glucostat allows maintenance of a high value of specific productivity (q_p) throughout the glucostat phase. The q_p value during glucostat phase of 30 g/l glucostats (Fig. 6b) was comparable to q_p value during phase 1 of 30 g/l batch experiments (Fig. 4b). Similarly, the MW_{HA} in the 30 g/l glucostats is comparable to the peak MW_{HA} obtained during 30 g/l batch fermentations.

Similar to the observations in the batch experiments, MW_{HA} showed a decreasing trend during the post-glucostat phase. MW_{HA} maintained during glucostat phase and at the end of post-glucostat phase is as shown in Table S5 (Supplementary data). The decrease in MW_{HA} during post-glucostat phase also validates the batch fermentation data, illustrating the strong influence of glucose concentration on MW_{HA}. The decrease in MW_{HA} during post-glucostat phase was also correlated with a decrease in q_p (Fig. 6a, b). The decrease in q_p during transition from glucostat phase to post-glucostat phase can be explained by decreasing q_s and $Y_{HA/S}$ values along with an increase in $Y_{LA/S}$ (Table 2), corroborating the similar trends observed in batch fermentation (Table 1).

Effect of glucose concentration on bioprocess parameters and MW_{HA} in glucostats

We analyzed how different glucose concentrations affect q_s and $Y_{HA/S}$, thereby influencing q_p and MW_{HA} in the glucostat experiments. Figure 7 shows the comparison of bioprocess parameters — q_s, μ, $Y_{HA/S}$, and q_p for the three recombinant *L. lactis* strains in the 10 and 30 g/l glucostat fermentations. The 10 g/l glucostat supported higher q_s than the 30 g/l glucostat (Fig. 7a). This observation is in accordance with the one made by Papagianni et al. (2007) during glucostat studies with *L. lactis* cultures. They observed that the specific glucose uptake rate and the activities of key glycolytic enzymes are maximum at 10 g/l in glucostat cultures of *L. lactis*. We observed that the higher q_s in 10 g/l glucostat fermentation supported higher growth rates (Fig. 7b) and biomass yields (Table 2) when compared to 30 g/l glucostat fermentation. High substrate flux into biomass formation resulted in lower $Y_{HA/S}$ values in 10 g/l glucostat when compared to 30 g/l glucostat (Fig. 7c). This leads to lower q_p values in 10 g/l glucostat fermentation as compared to 30 g/l glucostat fermentation (Fig. 7d) which explains the lower MW_{HA} obtained in 10 g/l glucostat fermentation as opposed to 30 g/l glucostat fermentation (Fig. 5, Table S5). The correlation of MW_{HA} with q_s, μ, $Y_{HA/S}$, and q_p during glucostat fermentation is shown in Fig. 8. The inverse correlation observed for MW_{HA} with specific growth rate (Fig. 8a) is supported by chemostat studies with recombinant *L. lactis* cultures (Badle

Table 2 Comparison of specific glucose uptake rate and yield coefficients of HA, biomass, and lactate based on glucose uptake, between glucostat phase and post-glucostat phase of glucostat fermentations of *L. lactis* GJP2, *L. lactis* GJP5, and *L. lactis* GJP6 carried out at 10 and 30 g/l glucose concentration

Glucose concentration (g/l)	Strain	Glucostat phase				Post-glucostat phase			
		q_s g/(g.h)	$Y_{HA/S}$ C-mol/C-mol	$Y_{X/S}$ C-mol/C-mol	$Y_{LA/S}$ C-mol/C-mol	q_s g/(g.h)	$Y_{HA/S}$ C-mol/C-mol	$Y_{X/S}$ C-mol/C-mol	$Y_{LA/S}$ C-mol/C-mol
10	GJP2	0.85	0.03	0.155	0.850	0.21	0.008	0.063	0.929
	GJP5	0.78	0.062	0.164	0.783	0.3	0.016	0.112	0.917
	GJP6	0.79	0.047	0.159	0.815	0.31	0.011	0.081	0.913
30	GJP2	0.53	0.075	0.090	0.825	0.37	0.005	0.039	0.960
	GJP5	0.57	0.103	0.118	0.835	0.37	0.010	0.053	0.952
	GJP6	0.64	0.093	0.094	0.806	0.29	0.010	0.089	0.926

Standard deviation in q_s and yield coefficients was < 5% of the corresponding values

et al. 2014). Earlier studies in our lab with fed-batch and chemostat cultures of recombinant *L. lactis* have shown that MW_{HA} correlates inversely with q_s (unpublished results). We also observed inverse correlations between MW_{HA} and q_s (Fig. 8b). A strong positive correlation is observed for MW_{HA} with $Y_{HA/S}$ (Fig. 8c) and q_p (Fig. 8d), which corroborates the inferences drawn for the decrease in MW_{HA} during phase 2 of the batch fermentations and post-glucostat phase.

Discussion

In this study, we observed that specific HA production rate (q_p) correlated strongly with MW_{HA}. To produce HA with different MW or to control MW_{HA} at a constant value during fermentation, q_p could serve as a suitable control parameter. The parameter q_p is influenced by the specific glucose uptake rate (q_s) and the fraction of glucose fluxed into the HA biosynthetic pathway ($Y_{HA/S}$). From Tables 1 and 2, it can be understood that the percentage drop in $Y_{HA/S}$ was higher when compared to percentage drop in q_s with progress of fermentation into phase 2 in case of batch fermentation and into post-glucostat phase during glucostat fermentation. So, decrease in glucose concentration affects resource allocation into HA pathway more than specific glucose uptake rate, which results in decrease of q_p and MW_{HA}. Thus, the variation in q_p and MW_{HA} values is more sensitive to the variation in $Y_{HA/S}$ than that in q_s.

The $Y_{HA/S}$ values are determined by carbon flux distribution to biomass formation ($Y_{X/S}$) and formation of by-products such as lactate ($Y_{LA/S}$). The latter two yield coefficients depend on process conditions, including the concentration of glucose. For example, in phase 2 of batch fermentations and in the post-glucostat phase, decreasing concentration of glucose resulted in higher values of $Y_{LA/S}$, thereby lowering the $Y_{HA/S}$ values (Tables 1 and 2). With reducing glucose concentration and uptake rate, increasing the lactate yield would be a natural strategy for the cell to improve its energy availability through substrate-level phosphorylation. Redon et al. (2005) reported downregulation of genes involved in transcription, translation, nucleotide biosynthesis, cell envelope biosynthesis, and cell division in *Lactococcus lactis* cultures during progression to nutrient depleted conditions in the later hours of batch fermentation. Redon et al. (2005) also observed glycolytic enzymes to be downregulated in the study. However, the measurement of specific activities of glycolytic enzymes indicated otherwise. Ercan et al. (2015) did a transcriptional analysis of *Lactococcus lactis* adapting to starvation conditions. They found the involvement of a transcriptional regulator (codY) in the downregulation of genes involved in transcriptional and cell division machinery, purine metabolism, and natural transformation. Hence, the shift in the carbon allocation from biomass and HA formation to

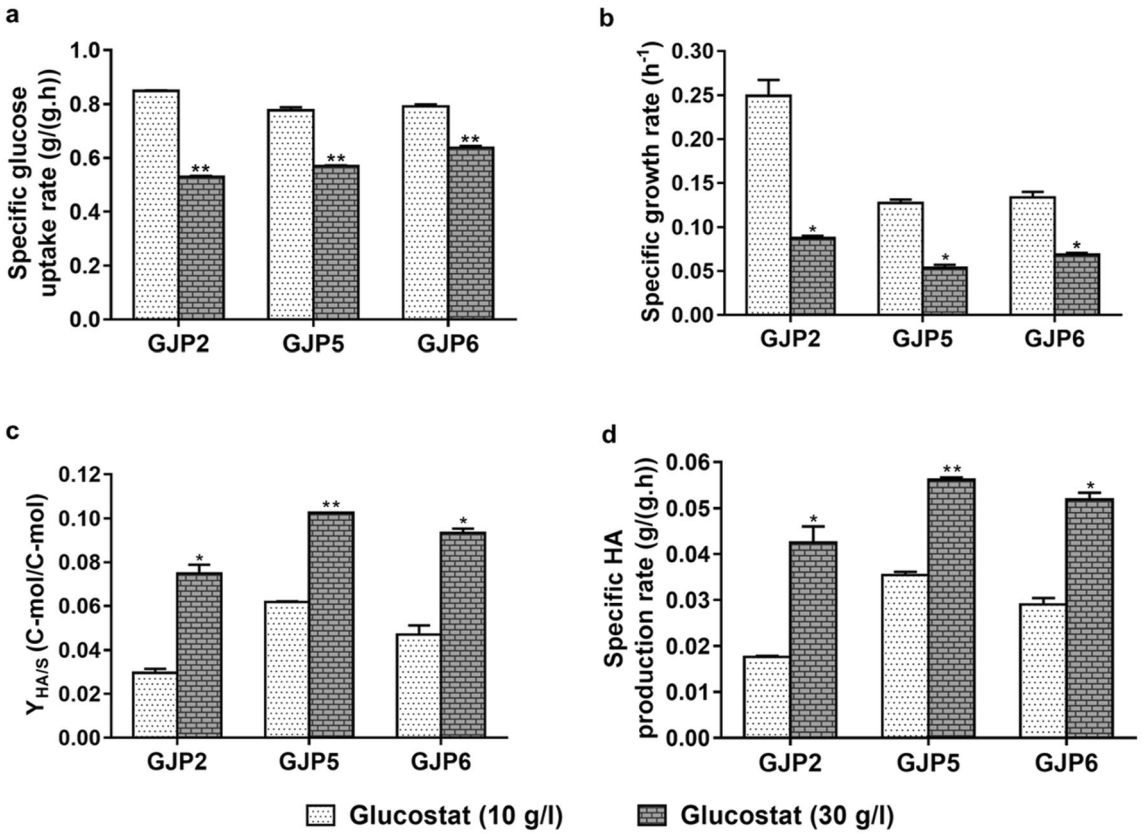

Fig. 7 Comparison of **a** specific glucose uptake rate, **b** specific growth rate, **c** $Y_{HA/S}$, and **d** specific HA production rate between glucostat experiments carried out at 10 g/l and 30 g/l glucose concentrations

glycolysis might be a result of transcriptional and allosteric regulation mechanisms. We also observed that the μ and $Y_{X/S}$ values were lower for the 30 g/l glucostats as compared to those of 10 g/l glucostats, resulting in higher values of $Y_{HA/S}$ (Table 2) and thereby leading to higher MW_{HA} in 30 g/l glucostats. For a given process condition, the $Y_{HA/S}$ values can also be enhanced by the overexpression of additional HA pathway genes as seen from the glucostat data (Table 2).

As shown in Tables S2 and S3 (Supplementary data), a range of genetic and process parameters can also be varied to obtain HA of different molecular weights. Metabolic engineering strategies for decreasing the glycolytic flux (Jin et al. 2016) or lactate formation (Kaur and Jayaraman 2016) have shown to enhance HA yield and MW_{HA}. Co-expression of HA pathway genes has also been a successful strategy to improve HA yield and MW_{HA} (Prasad et al. 2010; Chauhan et al. 2014). MW_{HA} can also be varied by manipulation of process parameters such as pH, agitation, aeration, initial glucose concentration, and temperature. However, the variation obtained in MW_{HA} is rather small in such studies (Tables S2 and S3). In this work, we have shown that $Y_{HA/S}$ values can be varied using simultaneous genetic (co-expression of HA biosynthetic genes) and process (glucose concentration) manipulations, thus enabling the production of HA over a 3-fold range of molecular weights.

While MW_{HA} is determined by $Y_{HA/S}$ (which strongly influences q_p), the maintenance of a constant molecular weight during fermentation is dependent on maintaining a high value of q_s and $Y_{HA/S}$. As can be discerned from Table 2, the q_s values are similar for a specific glucose concentration and are therefore controlled only by that process parameter. Therefore, glucose concentration serves as an ideal control parameter to sustain higher values of q_s and $Y_{HA/S}$ as demonstrated by the constant-MW HA production during glucostat fermentations in this work. Thus, by combining glucostat strategy with genetic and process parameters that offer tunability of MW_{HA}, we can modulate the production of constant-MW HA over a wide range of values.

Normally, glucose should be the only nutrient which influences values of q_s and $Y_{HA/S}$ during a glucostat strategy, for the production of constant-MW HA. But, when the reducing concentrations of other nutrients influence product formation rates, value of q_s and $Y_{HA/S}$ cannot be controlled by only controlling glucose concentration. Similar to glucostat strategy, nitrogen or phosphorus-controlled processes might also be able to maintain q_s and $Y_{HA/S}$, especially if the C/N ratio is

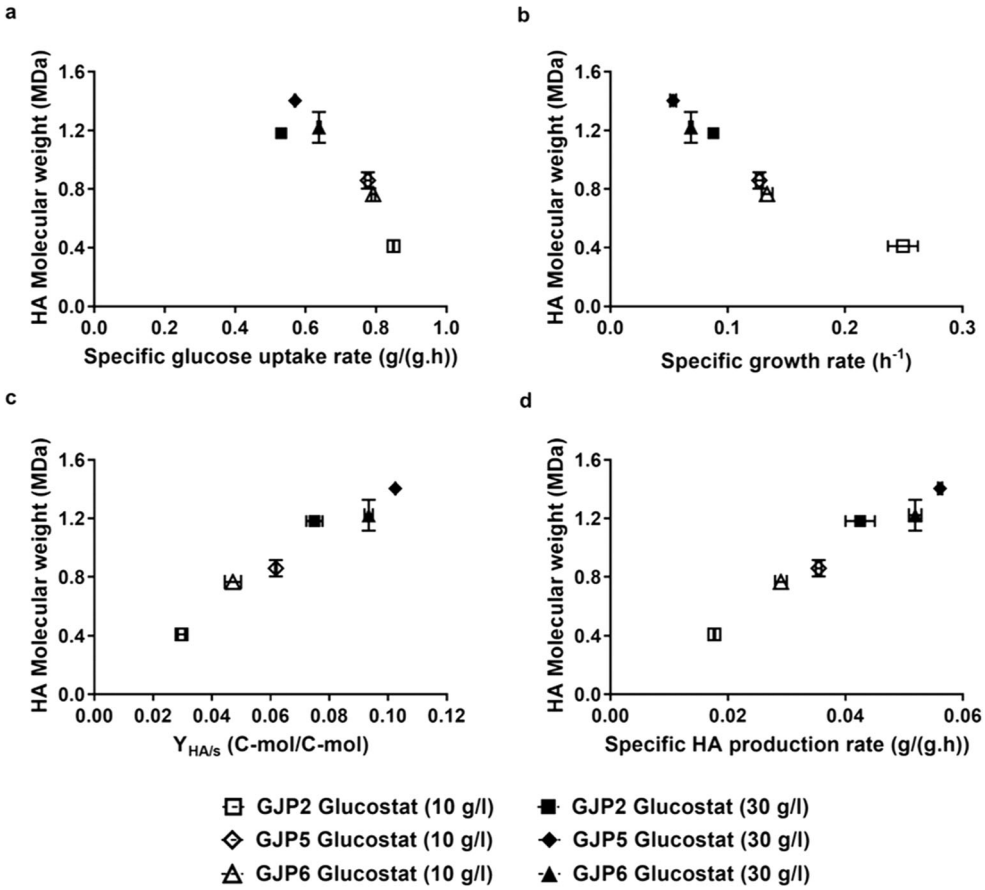

Fig. 8 Correlation of HA molecular weight with **a** specific glucose uptake rate, **b** specific growth rate, **c** $Y_{HA/S}$, and **d** specific HA production rate during glucostat experiments carried out at 10 g/l and 30 g/l glucose concentrations

kept constant. However, this is difficult to implement from an operational standpoint (especially for complex nitrogen sources), since it is difficult to measure or control nitrogen or phosphorous concentration online. Moreover, there is no indirect mechanism (such as lactate formation in case of glucose) which can indicate consumption of nitrogen or phosphorus. Therefore, a simpler strategy for maintaining glucose concentration and thereby q_s has been adopted in this study.

Molecular weight variation and its control during fermentation is a less explored area in literature on HA fermentation. This work has been an effort to understand MW_{HA} variation during fermentation, to develop strategies to control MW_{HA} during fermentation, and to employ the strategy developed to produce constant-MW HA over a range of molecular weights. The insights obtained from this study on molecular weight variation and the molecular weight control strategy developed in this work have significant implications for production of HA of desired molecular weights required for specific applications.

Acknowledgements The authors would like to acknowledge Indian Institute of Technology Madras for providing infrastructure to carry out this work and Ministry of Human Resource Development, Government of India for providing Fellowships to Sreeja Shanmuga Doss and Pandeeswari Jeeva.

Funding information The Department of Biotechnology (Ministry of Science and Technology, Govt. of India) provided funds to execute this project through Project No. BT/IN/Finland/30/GJ/2013.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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

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