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Hyaluronic acid production and molecular weight improvement by redirection of carbon flux towards its biosynthesis pathway

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ABSTRACT

Hyaluronic acid (HA) production in *Streptococcus zooepidemicus* competes for the carbon source along with biomass formation, lactate formation (via glycolysis) and pentose phosphate pathway (PPP). In our studies, increase in HA molecular weight was observed by redirecting the carbon flux towards HA biosynthesis pathway by partially inhibiting the glycolytic pathway. Batch bioreactor (1.2 L) studies showed that with the addition of 25 μM sodium iodoacetate, 5 g/L tryptophan and 10 g/L pyruvate, which are glycolytic inhibitors, HA molecular weight increased to 3.2, 3.2 and 3.1 MDa respectively compared to control run (2.4 MDa). Yield coefficients $Y_{HA/S}$ and $Y_{LA/S}$ showed inverse relationship, indicating competition for glucose between HA and lactic acid formation. Addition of 5 g/L glutamine along with 25 μM sodium iodoacetate also increased the HA concentration to 5.0 g/L from 2.0 g/L in control run. Metabolic flux analysis studies show that concentration and molecular weight of HA is increased by decreasing carbon flux towards glycolysis and PPP and increasing carbon flux towards HA precursor formation. It was observed that specific growth rate of the cells correlated positively to the specific HA production rate and negatively to the molecular weight of HA produced. Addition of antioxidant tannic acid also increased molecular weight to 3.0 MDa.

1. Introduction

Hyaluronic acid (HA) is an unbranched glycosaminoglycan composed of disaccharides of glucuronic acid and N-acetylglucosamine joined in an alternate fashion [1]. It is a high molecular weight biopolymer. HA forms highly viscous solutions in aqueous media allowing it to be used for various medical applications [2].

HA is found in the connective tissues of animals (such as, rooster comb) and in capsular form in *Streptococcus* bacteria belonging to Lancefield classification group A or C. HA is produced by extraction from rooster combs for commercial purposes. However, due to recent outbreaks of viral diseases from avian sources, there is a prejudice on the usage of poultry derived products for medicinal purposes. Therefore, microbial production of HA from *Streptococcus zooepidemicus* is a more attractive option, which also has comparatively reduced purification costs [1].

Recent reports on microbial production of HA include development of recombinant GRAS (Generally Recognized As Safe) strains for HA production. These studies include recombinant strains of *Bacillus subtilis* [3–5], *Escherichia coli* [6,7] and *Lactococcus lactis* [8–11]. The major problem with the recombinant strains is the low yield and low molecular weight of the HA produced compared to the HA produced by *S. zooepidemicus*.

So far, methods for HA production have emphasized mainly on the environmental or culture conditions for cell growth and HA formation rather than focusing on cellular metabolism and its regulation for obtaining higher yield and molecular weight [12–19]. The biosynthesis of HA faces a stiff competition between glycolytic pathway and cell wall synthesis [1,18]. It has also been reported that the molecular weight of HA is controlled by the concentration of the precursor UDP-N-acetylglucosamine which is limiting compared to the other precursor UDP-glucuronic acid [20]. Consequently, to increase the productivity and the molecular weight of HA, a balanced flux of these precursors towards HA biosynthesis is required.

The current study investigated the effect of redirecting the carbon flux on HA productivity and molecular weight. To redirect the fluxes, partial inhibition of the glycolytic pathway was done by the addition of iodoacetate, tryptophan and pyruvate and strengthening of UDP-N-acetylglucosamine formation by the addition of glutamine. Reducing the depolymerization of HA polymer chain length was by the addition of antioxidants like salicylic acid, oxalic acid and tannic acid. Iodoacetate is an irreversible inhibitor of glyceraldehyde 3-phosphate dehydrogenase enzyme of the glycolysis pathway [21]. Tryptophan is reported to be an inhibitor of pyruvate kinase enzyme [22,23]. Pyruvate is known to reduce the overall

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activity of glycolytic enzymes [24,25]. Glutamine acts as an amino group donor to fructose 6-phosphate in presence of amidotransferase enzyme which yields glucosamine 6-phosphate. Later on this forms UDP-N-acetylglucosamine is synthesized which is one of the precursors for HA formation. Addition of glutamine is known to increase HA production [14,26]. Reactive oxygen species are formed due to high aeration and agitation, they are known to depolymerise HA chains resulting in low molecular weight product [27]. Hence, usage of antioxidants can inhibit reactive oxygen species formation which can lead to higher molecular weight HA [14,27]. In this study, metabolic flux analysis was also carried out at different inhibition levels of glycolytic pathway to understand their effect on flux distribution in the metabolic network. Also, we have discussed the relationship among cell specific growth rate, HA molecular weight, HA and lactic acid yield.

2. Materials and methods

2.1. Strain and culture media

S. equi subsp. *zooepidemicus* ATCC 39920 was maintained in glycerol stock at −80 °C. It was revived by culturing the glycerol stocks onto brain heart infusion agar plates at 37 °C for 24 h before inoculation.

The transfer and reactor media used was a complex media with composition 30 g/L of glucose, 10 g/L of yeast extract, 1.5 g/L of $Na_2HPO_4{\cdot}12H_2O$, 0.5 g/L of $KH_2PO_4{\cdot}5H_2O$ and 0.5 g/L of $MgSO_4{\cdot}7H_2O$. Glucose and $MgSO_4$ were autoclaved separately and transferred aseptically to the sterilized media. Yeast extract from the same batch was used in all the experiments.

2.2. Cultivation

Colonies from brain heart infusion agar plate were inoculated into 10 mL brain heart infusion broth. The overnight incubated culture at 37 °C and 200 rpm was transferred into a 60 mL seed culture media in 250 mL Erlenmeyer flask and incubated (till optical density of at least 0.6 was obtained at 600 nm wavelength) at 37 °C and 200 rpm, before inoculating the reactor. The fermentation was carried out in an *in situ* sterilizable 2.4 L Bioengineering® reactor (Switzerland) with a working volume of 1.2 L. pH was maintained by 4 M NaOH solution; acidic solution was not required to maintain pH as initial pH of the media was 7.2 and it continuously dropped with the formation of organic acids. A control run was carried out to compare the results of the reactor runs. The control run is the reactor run with complex media containing 30 g/L glucose, aeration rate of 1vvm, pH maintained at 7.0, impeller agitation speed of 400 rpm and temperature controlled at 37 °C. All the reactor runs were repeated three times. The error bars in all the figures represent the standard deviation from the average values.

2.3. Feeding of inhibitors and amino acids

The range of concentration of all the additives used was determined previously in shake flask studies. Additives in excessive concentrations led to very poor biomass and HA production (data not shown). Sodium iodoacetate, pyruvate, amino acids and the antioxidants (tannic acid, oxalic acid and salicylic acid) were filter sterilized using 0.22 μm syringe filter and were added to the bioreactor aseptically through feeding bottle having silicon tubing with hypodermic needle at the end. Feed bottles, silicon tubing and hypodermic needles were autoclaved prior to use. The flow rate of the feed was controlled by a peristaltic pump.

2.4. Analytical methods

2.4.1. Measurement of biomass

For measurement of biomass, culture samples were treated with 1 volume of 0.1% SDS for 10 min to strip cells of capsular HA [28] and then centrifuged at 10,000 rpm for 10 min to pellet HA free cells. The supernatant was used to estimate HA concentration. The pellet was resuspended in 0.9% NaCl solution. The optical density of the resulting suspension was analysed at a wavelength of 600 nm (OD_{600}) for biomass using Jasco V550 UV–vis spectrophotometer for biomass estimation. Correlation between culture absorbance at OD_{600} and dry cell weight was found to be: 1 OD_{600} = 0.4 g/L dry cell weight. The supernatant was used for estimating HA and other metabolites.

2.4.2. Measurement of glucose and other metabolites

HA was estimated using the modified carbazole assay [29]. HA standards used were obtained from Calbiochem (San Diego, USA).

Glucose was estimated by the enzymatic glucose oxidase-peroxidase Merkotest method (Merck Specialties Pvt., Ltd., India). Lactate was estimated using HPLC (Shimadzu, Japan) fitted with a Phenomenox Rezex 300 mm × 7.8 mm column maintained at 50 °C. The mobile phase used was 5 mM H_2SO_4 with 0.6 mL/min flow rate. The detector used was diode array detector at a wavelength of 210 nm (Shimadzu, Model SPD-M20A, Japan).

2.4.3. Estimation of molecular weight

HA molecular weight was estimated using HPLC (Shimadzu, Japan) with gel permeation column Shodex OHpak 13u SB-805 HQ 500A-600 × 8 mm maintained at 30 °C. The mobile phase used was 0.1 M sodium nitrate at a flow rate of 1.0 mL/min. Refractive index detector was used to analyse the samples. Polyethylene oxide samples (0.6–4 MDa) obtained from Sigma–Aldrich (USA) was used as molecular weight standards.

2.5. Calculation of specific rates

Specific growth rate (μ) was calculated from the slope of the regression line in a plot between ln (X/X_0) and time during the exponential growth phase (where X and X_0 are the biomass at time "t" and at the beginning of the exponential phase, respectively, measured in g cell L^{-1}). The specific productivity (q_P) (g product g $cell^{-1}$ h^{-1}) was calculated from the discrete time course measurement using the following formula:

$$q_P = \mu \cdot \frac{\Delta P}{\Delta X}$$

where $\Delta P/\Delta X$ is the slope of the regression line for the plot between product concentration (P) and biomass (X) in the exponential phase. Similar equation was used for calculating the specific uptake rate of glucose.

2.6. Metabolic flux analysis

The stoichiometric equations of *S. zooepidemicus* were adapted from the equations provided by Jagannath and Ramachandran [25]. The model is an over-determined system of equations consisting of 33 equations and 34 variables with 7 metabolites measured during experimental study and final solution was achieved using Least squares method. Metabolites measured are glucose uptake rate, rate of formation of biomass, lactic acid, formic acid, acetic acid, hyaluronic acid and ethanol. Reactions considered for the flux analysis are shown in Table 1.

Table 1
Stoichiometric equations used in metabolic flux model for *Streptococcus zooepidemicus*.

Flux no.	Stoichiometric equations for *Streptococcus zooepidemicus*
Glycolysis and pyruvate metabolism	
[V1]	Glucose 6-phosphate → fructose 6-phosphate
[V2]	ATP + fructose 6-phosphate → ADP + 2 DHAP
[V3]	DHAP + ADP + phosphate + NAD(+) → phosphoenolpyruvate + H(2)O + ATP + NADH + H(+)
[V4]	ADP + phosphoenolpyruvate → ATP + pyruvate
[V5]	Pyruvate + NADH + H(+) → (S)-lactate + NAD(+)
[V6]	Pyruvate + CoA → acetyl-CoA + formate
[V7]	Pyruvate + CoA + NAD(+) → acetyl-CoA + NADH + CO(2)
[V8]	Pyruvate + phosphate + ADP + O(2) + H(2)O → acetate + ATP + CO(2) + H(2)O(2)
[V9]	Acetyl-CoA + 2 NADH + H(+) → ethanol + CoA + 2 NAD(+)
Pentose phosphate pathway	
[V10]	Glucose 6-phosphate + 2 NADP(+) + H(2)O → ribose 5-phosphate + CO(2) + 2 NADPH + 2 H(+)
[V11]	3 ribose 5-phosphate → 2 fructose 6-phosphate + DHAP
HA production pathway	
[V12]	Fructose 6-phosphate + L-glutamine + acetyl-CoA + UTP → UDP-N-acetyl-glucosamine + L-glutamate + CoA + 2 phosphate
[V13]	Glucose 6-phosphate + UTP + NAD(+) → UDP-Glucuronic acid + 2 phosphate + NADH + H(+)
[V14]	UDP-N-acetyl-glucosamine + UDP-Glucuronic acid + 2 ATP + 2 NAD(+) → HA + 2 ADP + 2 UTP + 2 phosphate + 2 NADH + 2 H(+)
Nucleotide synthesis	
[V15]	Ribose 5-phosphate + 4 ATP + adenosine → ATPN + 4 ADP + pyrophosphate
[V16]	Ribose 5-phosphate + 4 ATP + uridine → UTP + 4 ADP + pyrophosphate
[V17]	Ribose 5-phosphate + 4 ATP + guanidine → GTP + 4 ADP + pyrophosphate
[V18]	UTP + L-glutamine + ATP → CTP + L-glutamate + ADP + phosphate
[V19]	ATP + NADPH → dATP + NADP(+)
[V20]	GTP + NADPH → dCTP + NADP(+)
[V21]	CTP + NADPH → dGTP + NADP(+)
[V22]	UTP + MTHF + 2 ATP + NADPH → DTTP + DHF + 2 ADP + NADP(+) + pyrophosphate
Macromolecules and biomass	
[V23]	2 UDP-N-acetyl-glucosamine + phosphoenolpyruvate + 3.5 L-alanine + L-glutamate + L-lysine + 8 ATP + NADPH → PG + 2 UTP + 8 ADP + 7 phosphate + NADP(+)
[V24]	UDP-glucose + 3 DHAP + 3 NADH + 5 ATP → TEIC (techoic acid) + 3 NAD(+) + 5 ADP + UDP
[V25]	23.9 acetyl-CoA + 2.1 DHAP + 0.65 UDP-glucose + 43.5 NADPH + 24.3 ATP + 2.1 NADH → LIP (lipid) + 23.9 CoA +43.5 NADP(+) + 24.3 ADP + 2.1 NAD(+) +0.65 UTP + 1.1 pyrophosphate + 2.2 phosphate + 2.1 H(2)O
[V26]	UDP-N-acetyl-glucosamine + 2 glucose 6-phosphate + 2 NADPH + 3 ATP → AWALL (antigenic wall) UTP + NADP(+) + 3 ADP + 2 pyrophosphate
[V27]	100 AA (amino acid) + 430.6 ATP → 100 PROT + 430.6 ADP + 430.6 phosphate
[V28]	29 dATP + 29 dTTP + 21dGTP + 21 dCTP + 137.2 ATP → 100 DNA + 137.2 ADP + 137.2 phosphate + 100 pyrophosphate
[V29]	26.2 ATPN + 21.6 UTP + 32.2 GTP + 20 CTP + 40 ATP → 100 RNA + 40 ADP + 40 phosphate + 100 pyrophosphate
[V30]	86.7 PROT + 2 PG + 1.6 TEIC + 0.7 AWALL + 1.1 DNA + 7.6 RNA + 0.5 LIP → BIOMASS
Energy and maintenance reactions	
[V31]	ATP → ADP + phosphate
[V32]	NADH + 0.5 O(2) → NAD(+) + H(2)O
Glucose transport through membrane	
[V33]	GLUCext + phosphoenolpyruvate → glucose 6-phosphate + pyruvate

(adapted from Jagannath and Ramachandran [25]).

3. Results

3.1. Effect of addition of glycolytic pathway inhibitors

3.1.1. Effect of addition of Iodoacetate

Iodoacetate is a specific and irreversible inhibitor of glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. It inhibits pyruvate kinase and L-lactate dehydrogenase only at millimolar concentrations [21]. Iodoacetate is also reported to completely inhibit glycolysis at 50 μM concentration [30]. Therefore, in the current study only low concentrations of iodoacetate (up to 25 μM) were used. The biomass concentration was same at 10 μM and 25 μM iodoacetate concentration compared to control run (2.6 g/L) (Fig. 1). However, lactic acid production significantly decreased to around 10 g/L at both concentrations of iodoacetate compared to 18.6 g/L in control run (Fig. 2). *Streptococcus* being part of the lactic acid bacteria family is prone to convert most of the glucose consumed to lactic acid *via* glycolysis pathway. Thus, decreased lactic acid production is an indicator of diminished glycolytic functioning. This led to increased HA production from 2 g/L (in control) to above 3 g/L upon addition of 10 and 25 μM iodoacetate concentrations (Fig. 3). Molecular weight of HA increased with increasing concentration of iodoacetate. And it was 2.4, 2.7 and 3.2 MDa for the control run, 10 μM and 25 μM of sodium iodoacetate concentrations respectively (Fig. 4). The inhibitor effect also corresponded with a decrease in specific growth rate from 0.42 h^{-1} in control run to 0.39 h^{-1} and 0.25 h^{-1} at 10 μM and 25 μM iodoacetate concentrations respectively (Fig. 5). Thus weakening of the glycolytic pathway can be attributed to diversion of carbon flux away from glycolysis to HA production pathway. This was further confirmed by metabolic flux analysis studies in which addition of iodoacetate showed decrease in carbon flux for formation of fructose 6-phosphate (Frc6P). Frc6P further goes into glycolysis to give lactic acid as the end product. With increased iodoacetate concentration the flux of Frc6P formation decreased from 95.0% in the control run to 77.5% (25 μM iodoacetate) (Fig. 6). Also flux of carbon towards UDP glucuronic acid (UDPGlc) formation increased with addition of increasing iodoacetate concentration (3.1% for control run, 3.2% for 10 μM iodoacetate and 4.7% for 25 μM iodoacetate). The increased carbon flux towards UDPGlc

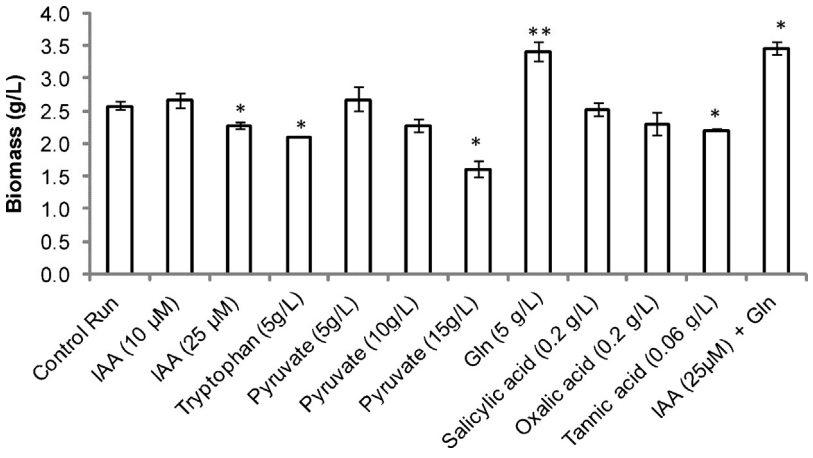

Fig. 1. Effect of various additives on biomass concentration (* signifies $p < 0.05$ and ** signifies $p < 0.005$, compared to control run).

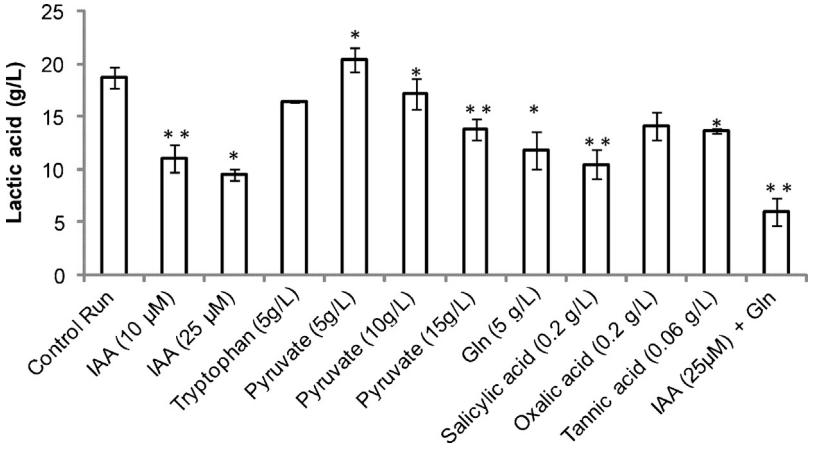

Fig. 2. Effect of various additives on lactic acid concentration (* signifies $p < 0.05$ and ** signifies $p < 0.005$, compared to control run).

only led to increased molecular weight of HA, but not its concentration.

3.1.2. *Effect of addition of tryptophan*

Tryptophan is a mild inhibitor of pyruvate kinase enzyme [22,23]. Also formation of one mole of tryptophan requires 5 moles of ATP [31]. Since HA production also requires ATP, the ATP saved in formation of tryptophan could be diverted towards HA production pathway. At 5 g/L tryptophan concentration, there was a slight reduction in biomass (2.1 g/L) and lactic acid (16.4 g/L) formation compared to control run but no significant increase in HA concentration (2.1 g/L) was observed (Figs. 1–3). The specific growth rate of the cells increased to 0.48 h^{-1} from 0.42 h^{-1} for the control run (Fig. 4). However, there was significant increase in molecular weight of HA (3.2 MDa) compared to control run (2.4 MDa). Literature reports [1,25] suggest that there is an inverse correlation of specific growth rate and molecular weight of HA produced. Such a correlation was not found in the case of addition of tryptophan

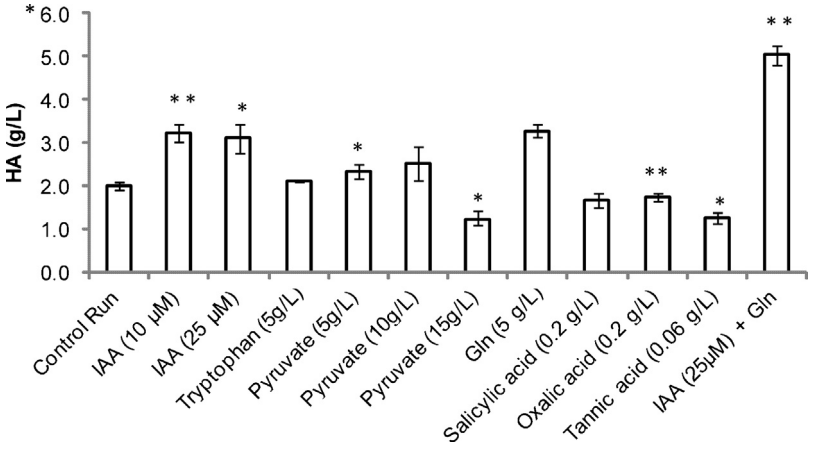

Fig. 3. Effect of various additives on Hyaluronic acid (HA) concentration (* signifies $p < 0.05$ and ** signifies $p < 0.005$, compared to control run).

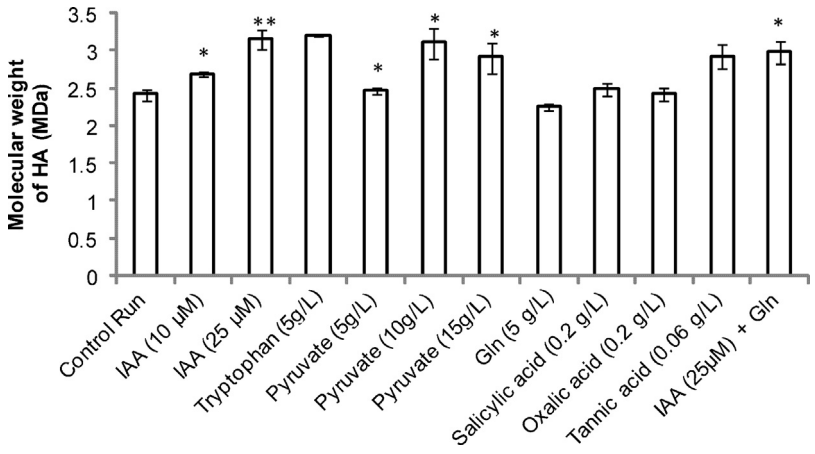

Fig. 4. Effect of various additives on Hyaluronic acid (HA) molecular weight (* signifies $p < 0.05$ and ** signifies $p < 0.005$, compared to control run).

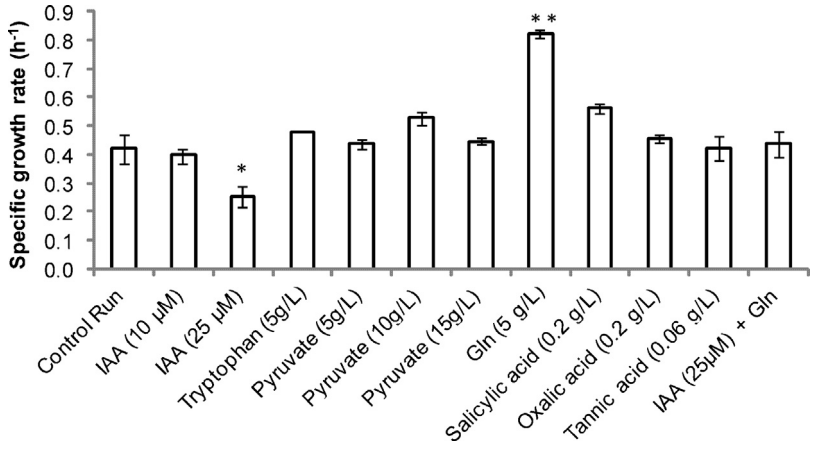

Fig. 5. Effect of various additives on specific growth rate (h^{-1}) (* signifies $p < 0.05$ and ** signifies $p < 0.005$, compared to control run).

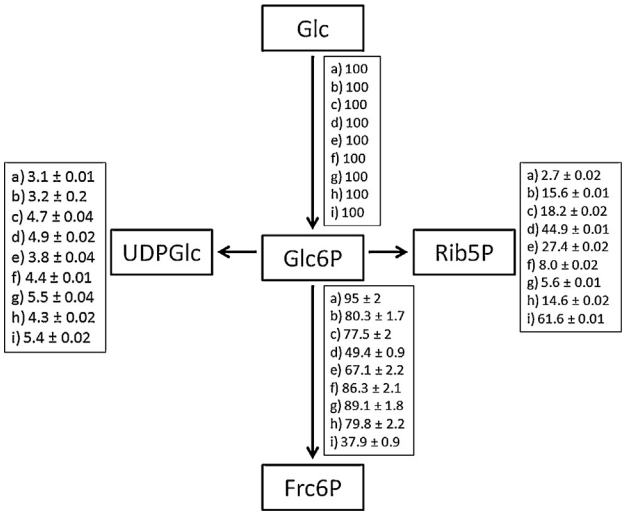

Fig. 6. Flux distribution at node Glucose 6-phosphate at the following conditions: (a) control run; (b) iodoacetate (10 μM); (c) iodoacetate (25 μM); (d) tryptophan (5 g/L); (e) pyruvate (5 g/L); (f) pyruvate (10 g/L); (g) pyruvate (15 g/L); (h) glutamine (5 g/L); (i) Iodoacetate 25 μM + glutamine (5 g/L). The p value for all fluxes is less than 0.005, except for UDP-glucuronic acid flux at (b) ($p < 0.05$) when compared to control run. The values of the fluxes are normalized with respect to 100 mol glucose/mol biomass/hour.

(Fig. 5). An explanation for the increase in molecular weight of HA by the addition of tryptophan could be because HA formation is a highly energy consuming process (using 3 ATP and 2 UTP molecules for each monomer of HA) [1] and tryptophan addition helped in conserving ATP and increasing the molecular weight of HA by diversion of ATP towards HA production pathway. It can be seen from Fig. 6, the carbon flux towards Frc6P decreased to 49.4% indicating inhibition of glycolysis to a great extent. Also a rise in UDPGlc formation flux to 4.8 was observed which corresponded with high molecular weight of HA (3.2 MDa). There was increased flux of carbon towards ribose 5-phosphate (Rib5P) formation, indicating intense competition for glucose between glycolysis, pentose phosphate pathway and HA synthesis.

3.1.3. *Effect of addition of pyruvate*

Pyruvate is known to reduce the overall activity of glycolytic enzymes [24,25]. Increasing pyruvate concentration by its addition in the media decreased the concentration of biomass formed (2.6, 2.2 and 1.6 g/L of biomass for 5, 10 and 15 g/L of pyruvate concentration respectively) compared to control run (2.6 g/L) (Fig. 1). This also corresponded with decreased lactate production at higher concentration of added pyruvate (20, 17 and 13 g/L of lactic acid for 5, 10 and 15 g/L of pyruvate concentration respectively) (Fig. 2). Thus pyruvate acted as an inhibitor of glycolysis pathway leading to reduced lactic acid concentrations. HA concentration and

molecular weight showed an optimum (2.5 g/L and 3.1 MDa) at 10 g/L pyruvate concentration (Figs. 3 and 4). Addition of pyruvate did affect glycolysis as expected. The specific growth rates observed were 0.43, 0.52 and 0.44 h^{-1} on addition of 5, 10 and 15 g/L pyruvate concentration respectively, which were higher than that of the control run (0.42 h^{-1}) (Fig. 5). The flux analysis studies showed lesser carbon flux towards Rib5P formation (8.0% and 5.6% for 10 and 15 g/L pyruvate concentration respectively). There was an increased flux towards UDPGlc for all pyruvate concentrations (Fig. 6). This corresponded with high molecular weight of HA produced (3.1 and 2.9 MDa for 10 and 15 g/L pyruvate concentration respectively). Also pyruvate is a known anti oxidant [32] and this property of pyruvate may also have helped increasing polymer chain length by detoxifying reactive oxygen species. In previous cases, addition of iodoacetate and tryptophan decreased the carbon towards Frc6P formation and led to high molecular weight of HA produced. This suggests interconnectivity of HA synthesis with glycolysis and pentose phosphate pathway.

3.2. Effect of addition HA pathway enhancer (glutamine)

Addition of glutamine is known to increase HA production by being an amino group donor for N-acetyl glucosamine formation [14,26]. In this case, biomass concentration was significantly higher but lactic acid formation was lower compared to control run (Figs. 1 and 2). This indicates that the glucose consumed was redistributed more towards formation cellular components like cell wall and less towards lactic acid formation. There was also an increase in HA concentration to 3.3 g/L indicating that addition of glutamine did enhance HA production pathway (Fig. 3). This result correlated with previous reports in literature [14,26]. However, there was no significant change in molecular weight compared to the control run (Fig. 4). A very high specific growth rate of 0.823 h^{-1} was observed by addition of glutamine, which is nearly twice that of the control run (Fig. 5). The flux analysis showed carbon flux decreased towards Frc6P formation and increased towards Rib5P formation and UDPGlc formation (79.8%, 14.6% and 4.3% respectively) compared to the control run (Fig. 6). Even though there was no increase in the molecular weight, there was an increase in HA concentration. Glutamine acts towards formation of UDP-N-acetylglucosamine (UDPNAG). The flux towards formation of UDPNAG had increased from 4% (in control run) to 5.4% (addition of glutamine) (data not shown). This might have led to balance in fluxes of carbon towards the precursors leading to higher HA concentration.

3.3. Effect of addition of anti oxidants

Reactive oxygen species are formed due to high aeration and agitator rates and are known to depolymerise HA chains to yield low molecular weight compounds [27]. Hence, usage of antioxidants (tannic acid, oxalic acid and salicylic acid) may inhibit reactive oxygen species formation leading to higher molecular weight HA [14,27]. For all the antioxidants added, there was no significant change in biomass production compared to the control run (Fig. 1). Salicylic acid addition showed decreased lactic acid formation but addition of none of the anti oxidants could increase HA concentration (Figs. 2 and 3). Molecular weight of HA was significantly enhanced to 2.9 MDa by the addition of tannic acid compared to molecular weight of 2.4 MDa obtained in control run (Fig. 4). In our study no significant increase in the molecular weight of HA was observed with the addition of salicylic acid, unlike reported by Zhang et al. [27] who achieved molecular weight improvement by 32%.

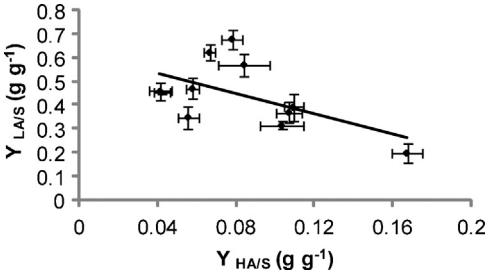

Fig. 7. Relationship between lactic acid yield ($Y_{LA/S}$) and HA yield ($Y_{HA/S}$).

3.4. Effect of addition of sodium iodoacetate and glutamine

After obtaining above results, various combinations of glycolytic inhibitors, glutamine and antioxidants were tried. It was observed that except for combination of sodium iodoacetate and glutamine, others led to poor biomass production (data not shown). Interestingly, least lactic acid production (6.0 g/L) was observed in this combination with highest biomass concentration (3.5 g/L) (Figs. 1 and 2). Also highest HA production of up to 5.0 g/L was observed (Fig. 3). This indicates that most of the glucose was fluxed away from lactic acid production to biomass and HA production. However, molecular weight of HA for the combination of sodium iodoacetate and glutamine (3.0 MDa) was not significantly different when only iodoacetate was added (3.2 MDa) (Fig. 4). This shows that glutamine addition has no effect on molecular weight of HA, but had an effect on HA production. This was supported by the metabolic flux analysis data also. The carbon flux towards formation of Frc6P was the lowest by the addition of sodium iodoacetate and glutamine together. The flux towards formation of Frc6P was 95.0, 77.5 and 37.9% in the control run, for iodoacetate (25 μM) and sodium iodoacetate (25 μM) + glutamine (5 g/L) respectively (Fig. 6). This diversion of carbon flux away from Frc6P formation and therefore glycolysis, probably led to high HA molecular weight as well as concentration.

4. Discussion

4.1. Competition for glucose between HA production and other metabolic pathways

Fig. 7 shows that yield of lactic acid per glucose ($Y_{LA/S}$) consumed is inversely related to yield of HA per glucose ($Y_{HA/S}$). *Streptococcus* being part of the lactic acid producing bacteria family produces lactic acid as the major product from glucose as the substrate. Reduction in flux of glucose from glycolysis using inhibitors led to higher HA formation. The competition for substrate between HA and other pathways is also reported by Chong et al. [1] and Liu et al. [17].

The metabolic flux analysis shows the glucose 6-phosphate is an important node in the metabolic pathway of glucose (Fig. 6). Glucose from this node can be directed to UDP-glucuronic acid formation, ribose 5-phosphate formation (pentose phosphate pathway) and fructose 6-phosphate formation (glycolysis and UDP-N-acetylglucosamine formation). It was observed that with decrease in carbon flux towards fructose 6-phosphate formation, there was higher HA production as well as higher molecular weight (as seen by addition of 10, 25 μM iodoacetate and combination of 25 μM iodoacetate with 5 g/L glutamine). Also, decreased carbon flux towards ribose 5-phosphate led to high molecular weight of HA (as seen at 10 and 15 g/L pyruvate concentration). Decreased carbon flux for the formation of Frc6P and Rib5P led to only high molecular weight of HA (as seen at 5 g/L tryptophan concentration) (Fig. 6). It can be concluded that redirecting glucose away from

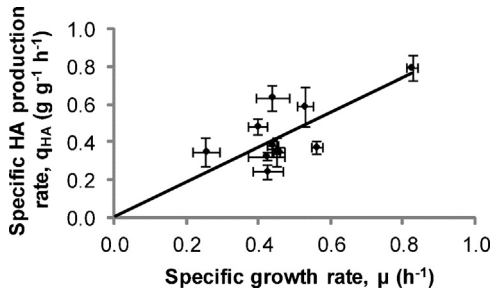

Fig. 8. Relationship between specific HA production rate, q_{HA} (h^{-1}) and specific growth rate, μ (h^{-1}).

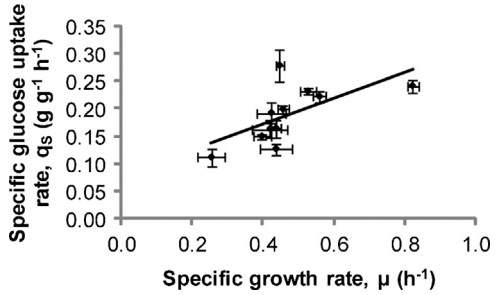

Fig. 9. Relationship between specific glucose uptake rate, q_S (h^{-1}) and specific growth rate, μ (h^{-1}).

glycolysis as well as pentose phosphate pathway was beneficial for HA production. An optimum balance between the supply of carbon towards HA synthesis, glycolysis and pentose phosphate pathway will lead to high HA concentration as well as high molecular weight.

4.2. Effect of specific growth rate on HA production

Specific growth rate is an important indicator of the cells state in a given environment. Lower the specific growth rate, the more stressful is the environment for the cell growth. From Fig. 8, it can be observed that the specific growth rate had a positive effect on the specific HA production rate (q_{HA}). Thus HA is a growth associated product in *S. zooepidemicus*. This could be linked to higher glucose uptake rate at higher specific growth rates (Fig. 9). More glucose uptake rate by the cell leads to more distribution through various metabolic pathways. Glucose, after satisfying the primary needs of a cell like glycolysis and cell wall components, overflows into HA synthetic pathway for increased production. Chong et al. [1] reported that the specific HA production rate is inversely proportional to the specific growth rate which is not in agreement with our results.

Molecular weight of HA showed inverse relationship with specific growth rate (Fig. 10). This finding was also observed by Chong et al. [1], Armstrong and Johns [12] and Jagannath and Ramachandran [25]. This shows that molecular weight and specific HA production rate behave differently with specific growth rate. Higher cell doubling time (low specific growth rates) allows the cell to continue elongation of HA chains. Also at lower specific growth rate in stressful environments, the cell focuses most of its resources towards HA chain elongation, which acts as a physical capsular barrier.

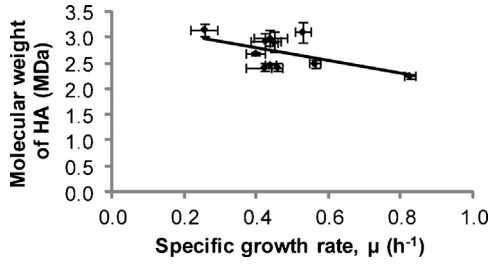

Fig. 10. Relationship between molecular weight of Hyaluronic acid (MDa) and specific growth rate, μ (h^{-1}).

4.3. Comparison of HA production with other studies

In this study, the highest molecular weight of HA, 3.2 MDa, was obtained with the addition of 25 μM sodium iodoacetate and tryptophan and a maximum HA yield of 5 g/L was obtained at 5 g/L glutamine concentration and 25 μM sodium iodoacetate together, using 3% glucose as carbon source. Im et al. [14] have reported the highest molecular weight of HA of 5.9 MDa with a yield of 6.94 g/L using 4% glucose as a carbon source. They used *Streptococcus* sp. ID 9102, a mutant strain which is hyaluronidase negative while the strain used in our study is a wild strain with an ability to synthesize hyaluronidase enzyme which lowers the molecular weight of HA. Rangaswamy and Jain [33] used wild *Streptococcal* strain and obtained HA yield of 0.1 g/g of sucrose compared to our yield of 0.16 g/g of glucose. Higher yield observed in this study is due to increased carbon availability for HA biosynthesis pathway. In our study no significant increase in the molecular weight of HA was observed with the addition of salicylic acid, unlike reported by Zhang et al. [27] who achieved molecular weight improvement by 32%.

5. Conclusions

The influence and control of glycolytic pathway on the molecular weight and the production of HA was examined and a detailed metabolic flux analysis has been carried out. Since HA synthesis pathway competes with glycolytic pathway and cell wall components synthesis for glucose, it is shown that with the addition of glycolytic inhibitors, increase in HA yield and molecular weight can be obtained. By metabolic flux analysis changes in the fluxes were observed at different levels of inhibition of glycolytic pathway. The analysis of experimental data and flux distribution values in bioreaction network showed that the fluxes at principal node of glucose 6-phosphate changed significantly under glycolytic pathway inhibition by diverting the carbon flux towards HA pathway and Pentose phosphate pathway, thus indicating that the node is not rigid and through genetic engineering higher yield of HA can be obtained. The addition of glutamine in the presence of glycolytic inhibitor further enhanced glucose flux towards the HA pathway. Inverse relationship between specific growth rate and HA molecular weight was observed. Competition for glucose was shown by the inverse relationship between lactic acid yield and HA yield.

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