



(51) International Patent Classification:

C12M 1/00 (2006.01)

C12R 1/46 (2006.01)

A61K 31/728 (2006.01)

(21) International Application Number:

PCT/IN2020/050447

(22) International Filing Date:

19 May 2020 (19.05.2020)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

201941025143

25 June 2019 (25.06.2019)

IN

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(81) Designated States (unless otherwise indicated, for every

kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every

kind of regional protection available): ARIPO (BW, GH,

GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— of inventorship (Rule 4.17(iv))

Published:

— with international search report (Art. 21(3))

— in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE

(54) Title: PROCESS FOR PRODUCTION OF HIGH MOLECULAR WEIGHT HYALURONAN IN A RECOMBINANT *LACTOCOCCUS LACTIS* USING ACETATE CO-UTILIZATION FED-BATCH STRATEGY(57) Abstract: The molecular weight of hyaluronic acid (HA) is a critical property which determines its usage in various biomedical applications. The invention pertains to a process for production of higher MWhA (3.4 MDa) throughout the fermentation time with high yield in anaerobic microbial fermentation with control of process parameter. The recombinant cocci for the production of hyaluronic acid is strain of *Lactococcus lactis* MKG6 throughout the fermentation time with high yield. The fermentation process is one of batch acetate pulse feed, batch process with acetate and glucose pulse feed, constant fed batch and pH feedback fed batch fermentation.

**PROCESS FOR PRODUCTION OF HIGH MOLECULAR WEIGHT
HYALURONAN IN A RECOMBINANT *LACTOCOCCUS LACTIS* USING
ACETATE CO-UTILIZATION FED-BATCH STRATEGY**

5 **FIELD OF INVENTION:**

The invention pertains to metabolic engineering with special reference to hyaluronan production by recombinant microbial fermentations.

BACKGROUND OF THE INVENTION:

10 Hyaluronan / Hyaluronic acid (HA), is a biopolymer having important biomedical applications, and which requires many cofactors for its biosynthesis. HA is a glycosaminoglycan made of alternative β -glycosidic linkages of glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc). The precursors for these moieties, UDP-GlcUA and UDP-GlcNAc, are produced in independent pathways from the common substrate, glucose-
15 6-phosphate. The synthesis of these precursors competes with several major metabolic pathways and also requires several important cofactors such as UTP, NAD⁺, and Acetyl-CoA. Therefore, apart from the redirection of fluxes from the competing pathways to the HA pathway, cofactor availability can also play a major role in the production and molecular weight of HA obtained through fermentation processes. HA is a ubiquitous anionic
20 polysaccharide, naturally present in all vertebrates, synthesized as a capsular polysaccharide by an integral membrane protein called *hyaluronan synthase (hasA)*. HA has many commercial biomedical applications and the molecular weight of HA (MW_{HA}) is a key determinant for its applications in cosmetics and healthcare. In general, high molecular weight HA is nonimmunogenic and non-toxic. High molecular weight HA is used as a visco-
25 supplement for treating osteoarthritis and for eye surgeries because of its greater resistance to degradation. Low molecular weight HA is more often used in wound healing, anti-cancer drugs, and cosmetic applications. Traditional routes for HA production include extraction from rooster combs and by fermentation of natural, and potentially pathogenic, microbial producers. In general, all organisms which have been pathway-engineered for HA
30 production have shown lesser productivity and also produce lower MW_{HA} compared with natural producers. Although there is substantial literature available on the process and metabolic engineering strategies for enhancement of HA production much less attention has been paid on strategies for MW_{HA} enhancement. Previous studies with recombinant *L. lactis*

strains had focused on different genetic strategies as well as process strategies to enhance MW_{HA} but had been unable to increase it beyond 1.6 MDa. The general range of MW_{HA} produced by these recombinant *L. lactis* strains varied from 0.6 - 1.6 MDa. This has also been generally true in case of other HA producing recombinant stains. Thus, there is a need
5 for the production of high MW_{HA} for medical applications.

Metabolic engineering has often targeted enzyme-coding genes in specific pathways, which need to be overexpressed (or knocked-out) for enhancement or redirection of fluxes towards metabolite overproduction. However, the limiting constraints for obtaining high product
10 yields or titers often lie in the enhanced availability of cofactors required for some of the steps in the targeted pathways. Many literature reports have focused on cofactor engineering for enhancing productivity and product yields, either by metabolic engineering or process strategies. Cofactors such as acetyl-CoA also act as precursors for a range of metabolites. Prior studies have achieved a 5-fold improvement in acetyl-CoA levels in *Escherichia coli*
15 cultures by over-expressing the rate-controlling enzyme (*pantothenate kinase*) thereby enhancing the acetate synthesis. Studies have been established a platform of yeast cell factory for enhanced production of cytosolic acetyl-CoA and its redirection towards products like α -santalene. There are disclosures that the triglycerides accumulation in green algae critically depends on their ability to divert carbon flow towards acetyl- CoA
20 biosynthesis. However, there are surprisingly few reports which have actually correlated an increase in intracellular concentration of these cofactors with enhanced product formation.

Acetyl-CoA is an important pathway node that connects the acetate metabolism and glycolysis with the HA biosynthetic pathway in *L. lactis*. Acetyl-CoA acts as a cofactor in
25 the step catalyzed by *acetyl transferase* in the pathway producing the limiting HA precursor, UDP-GlcNAc. Regulation of acetate metabolism and its assimilation as an alternative carbon source is still unexplored during *L. lactis* fermentation, although there are some reports in *E.coli* and other cultures have shown that overexpression of acetyl-CoA synthetase (ACS) in *E. coli* has led to better assimilation of acetate in cultures grown only on acetate.
30 Thus, there is still a need for enhancing the production of MW_{HA} using process strategies especially with acetate supplementation and co-utilization.

OBJECT OF THE INVENTION:

The object of the invention is for a process for the production of higher MW_{HA} (3.4 MDa) throughout the fermentation time with high yield by anaerobic microbial fermentation with the control of process parameter.

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DESCRIPTION OF FIGURES AND DRAWINGS:

Figure 1 depicts the construct of *Lactococcus lactis* MKG6

Figure 2 depicts the hyaluronic acid biosynthetic pathway in *L. lactis*. Linkage of HA pathway to acetate metabolism is shown through acetyl CoA, a key cofactor for the synthesis of the limiting precursor UDP-N-acetylglucosamine.

Figure 2 shows *In-silico* flux balance analysis using a genome-scale metabolic network of *L. lactis*. (a) Simulation showing a positive correlation between intracellular acetyl-CoA flux, UDP-GlcNAc flux, and experimental HA production rates (b) Simulation showing a positive correlation between intracellular acetyl-CoA flux, UDP-GlcUA flux, and experimental ethanol production rates.

Figure 3 illustrates the batch experiments for Hyaluronic acid production with *L. lactis* MKG6. Profiles of glucose consumption, acetate concentration, HA production, HA molecular weight (MW_{HA}) and precursor ratio; (a) and (c) Batch fermentation without acetate supplementation (B-Glc); (b) and (d) Batch fermentation with acetate supplementation (Ac-Pulse); Point of acetate pulse (18th hour) is represented by the black arrow in (b) and (d).

Figure 4 depicts the effect of co-supplementation of glucose and acetate on Hyaluronic acid production and acetate utilization. (a) Profile of precursor ratio (UDP-GlcNAc/UDP-GlcUA) and HA molecular weight (MW_{HA}); the black arrow represents the point of acetate and glucose pulse in the log phase of the batch cultures; (b) Glucose and acetate profiles, showing co-utilization during the fermentation process.

Figure 5 showing a constant feed rate fed-batch fermentation (GAc-CF-8:1). (a) Profile of precursor ratio (UDP-GlcNAc/UDPGlcUA) and HA molecular weight (MW_{HA}); the black arrow represents the start of co-feeding of acetate and glucose. (b) Fermentation profile of glucose consumption, acetate utilization, and HA production.

Figure 6 showing the glucose and acetate profiles for fed-batch processes with glucose feeding based on pH-feedback response (a) 21% feed concentration without acetate supplementation (FB-1x); (b) 21% feed concentration with acetate supplementation (FB-1x-

Ac); (c) 42% feed concentration with acetate supplementation (FB-2x-Ac); glucose feeding was started, along with acetate supplementation at 16th hour.

Figure 7 depicts the profiles for the molecular weight of Hyaluronic acid (MW_{HA}) produced in fed-batch experiments based on pH feedback.

5 **Figure 8** Thermo-gravimetric analysis of Hyaluronic acid.

ABBREVIATIONS:

B-Glc	Batch
Ac-Pulse	Batch with acetate pulse
GAc-Pulse	Batch with acetate and glucose pulse
GAc-CF-8:1	Constant feed Fed-batch
GAc-CF-2:1	Constant feed Fed-batch
FB-1x	pH feedback- fed batch fermentation
FB-1x-Ac	pH feedback- fed batch fermentation
FB-2x-Ac	pH feedback- fed batch fermentation
FB-3x-Ac	pH feedback- fed batch fermentation

DESCRIPTION OF THE INVENTION:

10 Accordingly, the invention pertains to a process for the production of higher MW_{HA} (3.4 MDa) throughout the fermentation time with high yield in anaerobic microbial fermentation with control of process parameter.

The recombinant cocci for the production of MW_{HA} 3.4 MDa is *Lactococcus lactis* MKG6.

15 *Lactococcus lactis* MKG6 expressed three heterologous HA-pathway genes obtained from the *has* operon of *Streptococcus zooepidemicus* in an *ldh*-mutant host strain, *L. lactis* NZ9020.

The titer of MW_{HA} 3.4 MDa obtained by using *Lactococcus lactis* MKG6 is 3.2 g/L in the fermentation condition of the process of the invention.

20 In one embodiment the invention discloses the flux distribution in the HA-precursor pathways in *L. lactis* strains, as the difference in the flux distribution to its precursor pathways causes significant variations in the amount of HA produced by different strains

and under different fermentation conditions. The flux distribution in the HA-precursor pathways in *L. lactis* strains was analyzed by a flux balance analysis (FBA).

The process parameter is of glucose or acetate for the increase in cofactors such as acetyl-CoA, UDP-GlcNAc, and HA production.

The process parameter is of glucose and acetate, for the increase in cofactors such as acetyl-CoA, UDP-GlcNAc, and HA production.

In one embodiment the invention discloses the increase in intracellular cofactors in HA biosynthetic pathway such as acetyl-CoA, UDP-GlcNAc.

The invention pertains to the anaerobic fermentation processes with the increase in intracellular cofactors such as acetyl-CoA, UDP-GlcNAc flux for increased HA productivity.

In one embodiment the anaerobic fermentation process of the invention is selected from the batch process with Ac-pulse or GAc-pulse.

In another embodiment, the anaerobic fermentation process of the invention is selected from the constant fed-batch process with high and low glucose to acetate ratio of 8:1 to 2:1.

In another embodiment, the anaerobic fermentation process of the invention is one of fed-batch process where 21% glucose feeding was initiated at the log phase of growth by the pH feedback mechanism in response to the base addition during the fed-batch process (FB-1x).

In another embodiment, the anaerobic fermentation process of the invention is one of a fed-batch process where 21% glucose feeding was initiated at the log phase of growth by the pH feed-back mechanism in response to the base addition during the fed-batch process. In addition acetate (5g/L) was pulsed once at the log phase (FB-1x-Ac).

In another embodiment, the anaerobic fermentation process of the invention is one of a fed-batch process where 42% glucose feeding was initiated at the log phase of growth by the pH

feedback mechanism in response to the base addition during the fed-batch process. In addition acetate (5g/L) was pulsed once at the log phase (FB-2x-Ac).

In another embodiment, the anaerobic fermentation process of the invention is one of a fed-batch process where 63% glucose feeding was initiated at the log phase of growth by the pH feedback mechanism in response to the base addition during the fed-batch process. In addition acetate (5g/L) was pulsed once at the log phase (FB-3x-Ac).

To increase acetyl-CoA levels, the anaerobic fermentation process was supplemented with acetate in batch and fed-batch processes. Further, the acetyl-CoA and the precursor availability were enhanced with acetate supplementation as a pulse input (Ac-Pulse) in the late log phase of the batch cultures and acetate utilization in this culture enhanced the HA concentration by 38% (Figure 3b). Although this led to only a marginal increase in acetyl-CoA, the peak MW_{HA} increased from 2.1 to 2.3 MDa (Figure 3d).

In addition, the invention discloses that acetate utilization takes place in *L. lactis* MKG6 cultures only in the presence of glucose, and to extend the acetate uptake phase, glucose was pulsed along with the acetate (GAc-Pulse) at the late log phase of the culture, when glucose concentrations decreased to around 5 g/l.

In one embodiment it is disclosed that the acetate uptake continued for a period of 12 hours after pulsing until the exhaustion of glucose (Figure 4b). A GAc-Pulse results in a 5-fold increase in acetyl-CoA levels compared to the Ac-Pulse, a 3-fold increase in the level of the HA-precursor UDP-GlcNAc and a 67% increase in MW_{HA} from 1.5 MDa to 2.5 MDa (Table 2). The invention further discloses that B-Glc and Ac-Pulse exhibited a decrease in MW_{HA} towards the end of the fermentation. The GAc-Pulse did not result in a decrease in MW_{HA} upon exhaustion of glucose. The MW_{HA} increased even after the glucose exhaustion. This increase is due to the accumulated level of acetyl-CoA and a favorable increase in precursor ratio (Figure 4a).

In one embodiment the invention discloses a constant fed-batch process for the production of high MW_{HA} wherein initially, fed-batch processes is with a constant feed rate having a fixed ratio of glucose to acetate. The glucose to acetate ratio was in the range of 8:1 to 2:1.

The feed rate of glucose and acetate (GAc-CF) in fed-batch fermentation process was 10ml/h constant. For high glucose to acetate ratio (GAc-CF-8:1), glucose and acetate were fed in the ratio of 1.6:0.2 g/h. The acetate utilization improved significantly (average utilization of 3.42 g/L) in the fed-batch phase, while the glucose concentrations remained constant around 2g/L (Figure 5b). The intracellular acetyl-CoA and HA precursors were comparable to the GAc-Pulse experiments, resulting in a similar HAMW (2.5 MDa) and a 23% increase in the HA titer (3.1 g/l) (Table 2). The MW_{HA} remained constant for a period of 30 hours, in contrast to the batch experiments which showed a decrease in MW_{HA}. The constant-feed fed-batch (GAc-CF-2:1), with lower glucose to acetate feed ratio (1.0:0.5 g/h), resulted in the accumulation of these substrates (Figure S4) and production of HA with lower titer (2.36 g/l) and less MW_{HA} (1.8 MDa) (Table 2).

The invention also discloses enhanced acetate utilization by maintaining a constant glucose concentration for *L. lactis* MKG6 cultures grown in hetero-lactic condition. The glucose uptake rate in the batch (B-Glc) cultures was 2.45 g/L/h. Concentrated glucose (21%) was fed, based on the pH-feedback response (FB-1x) and the net feed rate of glucose was 2.625 g/L/h during the FB-1x strategy. After the start of feeding, the average glucose and acetate concentrations were maintained at ~ 8 g/L and 2-3 g/l, respectively, for a period of 10 hrs (Figure. 6a). Compared to batch (B-Glc) cultures with only glucose as substrate, the MW_{HA} increased in the FB-1x experiment and was maintained in the range of 1.9–2 MDa till the end of fermentation for a period of 36 hours (Figure 7). The amount of HA produced also nearly doubled compared to batch fermentation (Table 2). The acetyl-CoA levels in FB-1x were much higher compared to all the batch experiments – viz. B-Glc, Ac-pulse, and GAc-pulse (Table 2).

Sodium acetate was pulsed along with the initiation of glucose feed in the fed-batch (FB-1x-Ac). The pH variations in these cultures were greatly reduced due to the buffering action of sodium acetate. The net feed rate of glucose feed was of 1.75 g/L/h and led to the exhaustion of glucose around the 40th hour. The acetate pulsing along with glucose feed in FB-1x-Ac resulted in a substantial increase in acetyl-CoA as well as the precursor UDP-GlcNAc and a higher MW_{HA} (2.31 MDa; P-value ~ 0.006), compared to the FB-1x experiment (Table 2). Further, feed glucose concentrations were doubled (to 42%) in the FB-2x-Ac conditions, keeping the same level of acetate pulsing (5 g/l). This resulted in

doubling the acetate uptake, a marginal increase in acetyl-CoA level and a significant increase in the level of the precursor UDP-GlcNAc (Table 2). The MW_{HA} increased by a further 50% to nearly 3.4 MDa (P-value ~ 0.003) (Table 2). The enhanced feed rate maintained the average glucose concentration at ~ 10g/L for a period of 15 hours (Figure 6c). The MW_{HA} was maintained at a high value throughout the fed-batch process (Figure 7). The precursor ratio increased gradually to a value close to unity, thereby enhancing the final average MW_{HA}. Further increase in the glucose feed rate with 63% glucose feed resulted in accumulation of glucose, lower values of acetyl-CoA (~ 276 nmol/g) and, consequently, a lower MW_{HA} (2.55 MDa), in comparison to the FB-2x-Ac fed-batch experiment (Table 2).

EXAMPLES:

Example 1: Construction of the recombinant *L.lactis*

The recombinant strain *L.lactis* MKG6 (Kaur and Jayaraman 2016), constructed by transformation of the plasmid pSJR6 (Figure E1) in *L. lactis* NZ9020 (*ldh* mutant version of *L. lactis* NZ9000) was employed in this study for HA production. *L. lactis* NZ9020 procured from NIZO, Netherlands, is a lactate dehydrogenase (*ldh*) mutant strain, in which two out of three *ldh* genes (*ldhX* and *ldhB*) have been knocked out (Bongers et al., 2003). The pSJR6 plasmid (Prasad et al. 2012) contains the heterologous *hyaluronan synthase* (*hasA*), *UDP-Glucose dehydrogenase* (*hasB*) and a bifunctional enzyme, *glucosamine-1-phosphate acetyltransferase / N-acetylglucosamine-1-phosphate uridylyltransferase* (*hasD/glmU*) genes sourced from *Streptococcus zooepidemicus* and overexpressed using the nisin-inducible NICE expression system (Mierau and Kleerebezem 2005). Stocks of *L. lactis* MKG6 strains were stored in 60% glycerol at -80°C. Chloramphenicol (10µg/ml) and tetracycline (2µg/ml) (Sigma Aldrich, India), were used as selection markers for the *L. lactis* MKG6 cultures. Fermentation experiments were carried out using a culture medium (HiMedia, India) composed of the following components (g/L): Yeast extract (5), brain heart infusion (5), KH₂PO₄ (0.5), K₂HPO₄ (1.5), MgSO₄·7H₂O (0.5), and ascorbic acid (0.5). Glycerol stocks were grown overnight in 50 ml medium, which is then sub-cultured in 100 ml medium to prepare the seed culture as inoculum for bioreactor studies.

Example 2:

1. Fermentation

Fermentation experiments were carried out using a culture medium (HiMedia, India) composed of the following components (g/L): Yeast extract (5), brain heart infusion (5), KH_2PO_4 (0.5), K_2HPO_4 (1.5), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5), and ascorbic acid (0.5).

- 5 Batch experiments were carried out in a 2.4L bioreactor (KLF-2000, Bioengineering, Switzerland) with 1.2 L of culture volume (including 100 ml inoculum seed culture) and 30 g/L initial glucose concentration. HA production was induced at 0.6 OD_{600} by addition of Nisin (2 ng/ml). Concentrated sodium acetate (HiMedia, India) was separately sterilized and used for pulsing experiments. Agitation, pH, temperature and dissolved oxygen were kept
- 10 constant throughout the process at 200 rpm, pH 7, 30°C and 0% (anaerobic), respectively. Fed-batch strategies (Table 1) were performed in a 3.6L bioreactor (KLF 2000, Biojenik Engineering, India) with the initial working volume (including 100 ml inoculum seed culture) of 1.6L respectively. Acetate production was significantly less compared to the formate synthesis. The amount of base consumption was estimated during the time course
- 15 of fermentation in batch studies. The change in formate production was positively correlated with the base consumption profile. The change in glucose utilization (ΔG) correlated linearly with the change in formate production (ΔF).

Table 1 Fermentation strategies employed for enhancing HA molecular weight

Experiment ID	Process Strategy	Total process time (h)	Acetate Supplementation	Glucose supplementation
B-Glc	Batch	27	-	-
Ac-Pulse	Batch with acetate pulse	35	10 g/L was pulsed at the 18 th hour	-
GAc-Pulse	Batch with acetate and glucose pulse	40	5 g/L was pulsed at the 14 th hour	10 g/L was pulsed at the 14 th hour
GAc-CF-8:1	Constant feed Fed-batch	50	0.2 g/h constant feed was initiated at the 14 th hour	1.6 g/h was pulsed at the 14 th hour
GAc-CF-2:1	Constant feed Fed-batch	48	0.5 g/h was pulsed at the 14 th hour	1.0 g/h was pulsed at the 14 th hour

FB-1x	pH feedback- fed batch fermentation	36	-	21% feed was started at the 16 th hour
FB-1x-Ac	pH feedback- fed batch fermentation	43	5 g/L was pulsed at the 16 th hour	21% feed was started at the 16 th hour
FB-2x-Ac	pH feedback- fed batch fermentation	45	5 g/L was pulsed at the 16 th hour	42% feed was started at the 16 th hour
FB-3x-Ac	pH feedback- fed batch fermentation	40	5 g/L was pulsed at the 16 th hour	63% feed was started at the 16 th hour

2. Flux Balance Analysis

Reconstructed Genome-scale Metabolic Network (GSMN) of *L. lactis* (Flahaut et al. 2013) with HA reactions (Badri et al. 2019) was used in this study. FBA was performed using
 5 COBRA toolbox v2.0 (Schellenberger et al. 2011) in the MATLAB R2012b (Mathworks Inc., USA). The metabolic constraints were modified using the estimated experimental rates obtained from batch experiments in Table 2.

Table 2 Estimated batch experimental constraints for Flux balance analysis

Experiment ID	Specific growth rate(h ⁻¹)	Specific substrate uptake rate (mmol/g.h)	HA productivity (mmol/g.h)	Lactate productivity (mmol/g.h)	Formate productivity (mmol/g.h)	Ethanol productivity (mmol/g.h)	Acetate productivity (mmol/g.h)
Anaerobic	0.33	15.1	0.70	0.7	14.75	10.89	4.5
Anaerobic	0.34	16.1	0.70	0.3	15.32	9.98	4.6
Aerobic	0.10	3.45	0.33	0.16	0	0.94	1.04
Aerobic	0.12	3.98	0.40	0.09	0	0.12	2.97
Aerobic	0.13	4.59	0.38	0.1	0	0.13	1.67

3. Fermentation Metabolites

All the solutions and buffers were prepared in ultrapure water and filtered in 0.2 µm PVDF membrane (Millipore, USA). The culture supernatant was diluted (10 times) and filtered through a 0.22µm membrane. Lactate, formate, glucose, acetate, and ethanol were estimated by ion-exchange chromatography using HPLC (Shimadzu, Japan) fitted with Phenomenex Rezex 300 × 7.8 mm column and the guard column (35×7.8 mm), maintained at 50°C. The mobile phase 5 mM H₂SO₄ was degassed on a bath sonicator and eluted at 0.6 ml/min flow rate. Retention times of lactate, formate, and acetate were 13.2 mins, 14.8 mins and 15.4 mins respectively, detected using diode array detector at 210 nm. Ethanol and Glucose were detected using a Shimadzu refractive index detector (RID) at retention times of 22.5 mins and 10.1 mins, respectively. Shimadzu LC solution software was used to integrate the peak area. Standard plots for these metabolites were developed individually to estimate their respective concentration in the fermentation broth. The slope of the analyte-specific standard plot was used to determine their concentrations from the area under the curve (AUC) of the respective metabolites.

Example 3:

1. Biomass Estimation

The culture sample was treated with an equal volume of 0.1 % sodium dodecyl sulphate (SDS) for 10 minutes to remove the capsular HA from cells (Gao et al. 2006) and then centrifuged at 10,000 rpm for 10 minutes to get HA free cell pellet. This was suspended in 0.9 % sodium chloride solution and optical density (OD600) was measured using UV-Visible spectrophotometer (Jasco Corp. V-550, Japan). The biomass concentration was then estimated from the calibration plot (Biomass DCW g/L = 0.376 * OD600).

2. HA Assay

HA was quantified by CTAB (Cetyl-tri-methyl-ammonium bromide) method described by Oueslati et al. (2014). The SDS treated broth samples were used directly for HA estimation. The Cetyl-tri-methyl-ammonium bromide (CTAB) reagent (2.5 g) was dissolved in 100 mL of 2% (w/v) sodium hydroxide. The SDS treated broth samples were used directly for assay. 50 µL of samples and 50 µL of 0.1 M phosphate buffer (pH 7) mixed in 96 well plates. The plates were incubated at 37°C for 15 minutes. Then, 100 µL of CTAB was added to each well and the plate was incubated for 10 minutes at 37°C. The samples were then mixed for

10 seconds to precipitate out HA. Absorbance at 600 nm was measured against the blank (0.1 M phosphate buffer) using UV-Visible spectrophotometer.

3. MW_{HA} estimation

5 The fermentation broth was first treated with an equal volume of 0.1 % SDS to remove the capsular HA from the cells (Chong and Nielsen, 2003). After centrifugation at 10,000 rpm for 10 minutes, the supernatant was taken and 4 volumes of ethanol were added and then incubated at 4°C for overnight. The precipitate thus obtained was collected by centrifugation at 10,000 rpm at 4 °C and re-dissolved in 1 volume of 0.2N NaNO₃ solution overnight. This
10 solution was then filtered using a 0.45 µm filter and used for estimating HA molecular weight. Phenomenex Polysep GFC- P 6000 column (300×7.8 mm) along with Phenomenex Polysep guard column (35×7.8 mm) fitted on a Shimadzu Prominence HPLC with Shimadzu RID was used for the analysis. Sample injection volume was 20µl. 0.2 M sodium nitrate filtered using 0.22 µm filter and degassed on a bath sonicator was used as mobile phase for
15 the analysis. Isocratic elution at 0.6 ml/min at ambient temperature was carried out. Shimadzu LC solution software was used to integrate the peak area. The calibration plot was constructed using peak position method using HA standards of viscosity average molecular weight, $M_v = 0.111$ MDa, 0.731 MDa, 1.59 MDa and 2.67 MDa with M_v values assumed to be equal to weight average MW (Armstrong and Johns, 1995). The calibration plot is shown
20 in Figure 14. The MW_{HA} measured using this method has a concentration effect, resulting in overestimation. Hence, the model used by Shanmuga Doss et al., 2017 was used to predict the true molecular weight.

Example 4: Sample processing for intracellular metabolites

25 1. Extraction of Acetyl-CoA

Samples were collected from the log phase of the culture broth and immediately processed for estimating the intracellular acetyl-CoA. Sample preparations were done using a modified version of the protocol reported in *Clostridium acetobutylicum* (Boynton et al., 1994). 10 ml of culture samples were centrifuged at 10000 rpm for 10mins at 4°C to remove the cell pellet.
30 An aqueous solution of 6% perchloric acid (800ul) was used to dissolve the pellet, providing the acidic environment to stabilize the intracellular acetyl-CoA. The entire pretreatment process should be carried out at 4°C with the use of dry ice. Then the cell disruption was carried out by probe-based sonication (QSONICA sonicators, New York) for 2 mins at the

amplitude of 65 MHz with the pulse on and off time of 2 seconds. Then the cells-free intracellular extracts were obtained by centrifugation at 10000 rpm for 2 mins at 4°C. Then the extracts were adjusted to pH 5-6 using 3M potassium carbonate (K₂CO₃). The precipitate was removed by centrifugation at 10000 rpm for 2 mins at 4°C. Then the supernatant was
5 stored in -80°C for reverse phase (RP-HPLC) analysis.

2. Estimation of Acetyl-CoA by RP-HPLC

RP-HPLC analysis was carried out using Phenomenex Luna C18 column (250×4.6 mm, 5 µm particle size) with Phenomenex guard column was fitted on a Shimadzu Prominence
10 HPLC along with PDA detector for this estimation. Two different solvents were used as mobile phase (A: 0.2 M sodium phosphate (pH 5.0); B: 0.25 M sodium phosphate (pH 5.0) with 200ml of acetonitrile). The gradient elutions were performed as described by Boynton et al.,1994. Elution was carried out at 35°C at 1 ml/min flow rate at 254 nm. Samples were pre-filtered before injection using 0.22µm membrane. Acetyl-CoA standards were dissolved
15 and diluted in 6% perchloric acid and the aliquots were stored in -80°C. The calibration curve for different concentrations of standard acetyl-CoA is shown in Figure 15.

3. Estimation intracellular UDP-HA precursors

Intracellular concentrations of UDP-N-acetyl glucosamine and UDP-glucuronic acid were
20 estimated using a modified procedure of cold ethanol extraction described by Ramos *et al.* 2001 (Ramos et al.,2001) followed by ion paired reverse phase HPLC of the lyophilized extract (IP-RP-HPLC). Culture samples (10ml) collected were immediately subjected to pre-treatment and the entire process has to be carried out on the ice. An equal volume of 0.1% SDS treatment was carried out to remove the capsular bound HA from cells. Then HA free
25 cells were pelleted at 4°C by centrifuging for 2 minutes at 10000rpm. The pellet obtained was re-suspended in ice-cold 2 ml of 50 mM MOPS buffer (pH 7) and sonicated for 10 minutes using a probe sonicator (QSONICA sonicators, New York). Sonication was carried out on the ice at an amplitude of 70 MHz for 5 mins at with the pulse on and off time of 2 seconds. Then 25 ml of ice-cold 70% ethanol was added to the sample and incubated at 4°C
30 with shaking for 20 minutes. Then the samples were centrifuged at 10000rpm at 4°C and the supernatant was powdered by lyophilization. This lyophilized sample was dissolved in mobile phase buffer A and filtered using 0.22 µm filter (EMD Millipore, US) before injection into HPLC.

4. Estimation of intracellular HA precursors

Phenomenex Luna C18 column (250×4.6 mm, 5 µm particle size) with Phenomenex guard column was fitted on a Shimadzu Prominence HPLC along with PDA detector were employed for IP-RP-HPLC. Gradient elution (Buffer A: 0.1M phosphate buffer (pH 6.4) with 8 mM tetra butyl ammonium hydrogen sulphate (TBAHS); Buffer B: 70 % buffer A and 30 % acetonitrile) was performed. The elution protocol described by Nakajima et al., 2010 was modified for better resolution of the precursors. The elution was carried out at 40°C at 0.3 ml/min flow rate at 254 nm. An injection volume of 20 µl was used. The samples were eluted according to the gradient scheme as follows: 100% A for 0-5 min, 0-100% B for 5-95 min, 100 % B from 95 to 110 min, 100-0% B between 110 and 115 min followed by 100% A until 180 min. The calibration plot of HA precursors (UDP-GlcNAc and UDP-GlcUA) are shown in Figure 16 and Figure 17 respectively.

Example 5: Extracellular metabolite Analysis

Lactate, formate, glucose, acetate, and ethanol were estimated using Ion-exchange chromatography (Kaur and Jayaraman 2016) using Phenomenex Rezex 300 × 7.8 mm column fitted in Shimadzu Prominence HPLC system connected with PDA and RID detector. The samples were diluted and filtered through a 0.22µm membrane before HPLC analysis.

Example 6: Characterization of HA polymer

The HA obtained by the fed-batch process was purified using the diafiltration technique (Rajendran et al. 2016). The final purified filtrate was lyophilized using freeze dryer (Lark, India) and then subjected to characterization.

Thermo-gravimetric analysis (TGA): 2g dried powder of purified and standard HA (Lifecore Biomedical, USA) was used for TGA. The thermal stability of the HA was tested using thermo-gravimetric analyzer TGA Q500Hi-Res (TA Instruments, USA). Heating was carried from 30 to 1000 °C at the rate of 20°C min⁻¹ under N₂ atmosphere.

¹H NMR spectroscopy: 5mg/mL of HA sample and standard were used for acquiring ¹H NMR spectra (Bruker Avance 500 MHz, USA). The analysis was performed at room temperature using Deuterated water (D₂O) as a solvent and the peaks were reported in parts

per million (ppm). These HA spectra were plotted using MNOVA software (Mestrelab Research, Spain).

ADVANTAGES:

- 5 1. Acetate is the cheap secondary substrate influences the intracellular concentrations of key cofactor, acetyl-CoA.
2. Fed-batch strategies co-utilises glucose and acetate facilitates high molecular weight HA production of 3.4 MDa.
3. The molecular weight profile seems to be maintained at high level throughout the
10 fermentation process without any significant decrease observed in batch studies.
4. The purified high molecular weight HA of the invention showed greater stability compared to the commercial HA standard (2.67 MDa) obtained from Lifecore Biomedicals, USA.

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The Claim:

1. A process for the production of hyaluronic acid of higher molecular weight (MW_{HA}) throughout the anaerobic microbial fermentation with a recombinant cocci wherein the acetate and glucose are pulse fed in the late log phase of fermentation process and wherein the cocci is a recombinant *Lactococcus lactis*.
2. The process as claimed in claim 1, wherein the fermentation process is one of batch acetate pulse feed, batch process with acetate and glucose pulse feed, constant fed batch and pH feedback fed batch process.
3. The process as claimed in claim 1, wherein the recombinant cocci is *Lactococcus lactis* MKG6.
4. The process as claimed in claim 3, wherein the recombinant *L. lactis* MKG6 harbors hyaluronan synthase (*hasA*), UDP-Glucose dehydrogenase (*hasB*) and a bifunctional enzyme, glucosamine-1-phosphate acetyltransferase/N-acetylglucosamine-1-phosphate uridylyltransferase (*hasD*)) genes.
5. The process as claimed in claim 1, wherein the MW_{HA} is 3.4 MDa.
6. The process as claimed in claim 1, wherein the glucose pulsing along with acetate enhances the final HA molecular weight by 63%.
7. The process as claimed in claim 1, wherein in the constant feed fed batch fermentation process, the glucose to acetate ratio is in the range of 8:1 to 2:1.
8. The process as claimed in claim 1, wherein in the pH feedback fed -batch fermentation process with 21% glucose feed, the average glucose concentration in the range of 7-9 g/L.

9. The process as claimed in claim 1, wherein in the pH feedback fed- batch fermentation process with 21% glucose feed the acetate concentration in the range of 2-3 g/l.
- 5 10. The process as claimed in claim 1, wherein in the pH feedback fed- batch fermentation process with 42% glucose feed with acetate pulse (5g/L) co-utilises glucose and acetate producing high HA molecular weight of 3.4 MDa.
- 10 11. The process as claimed in claim 1, wherein in the pH feedback fed- batch fermentation process with 42% glucose feed with acetate pulse (5g/L) HA titers is of 3.2 g/L.
12. The process as claimed in claim 1, wherein the cofactors such as acetyl-CoA, UDP-GlcNAc are increased in acetate utilization fed-batch processes.

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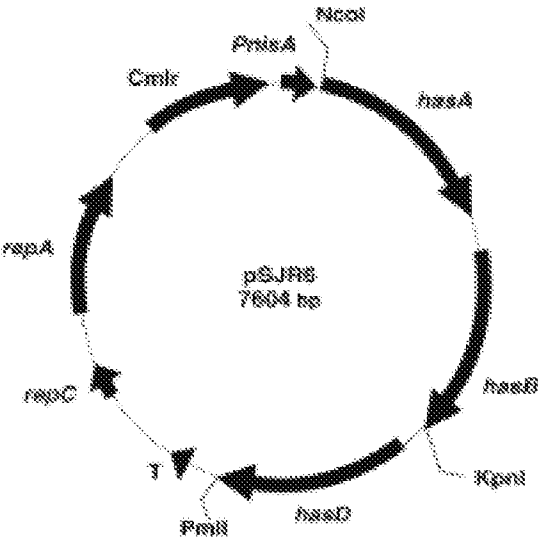


Figure 1

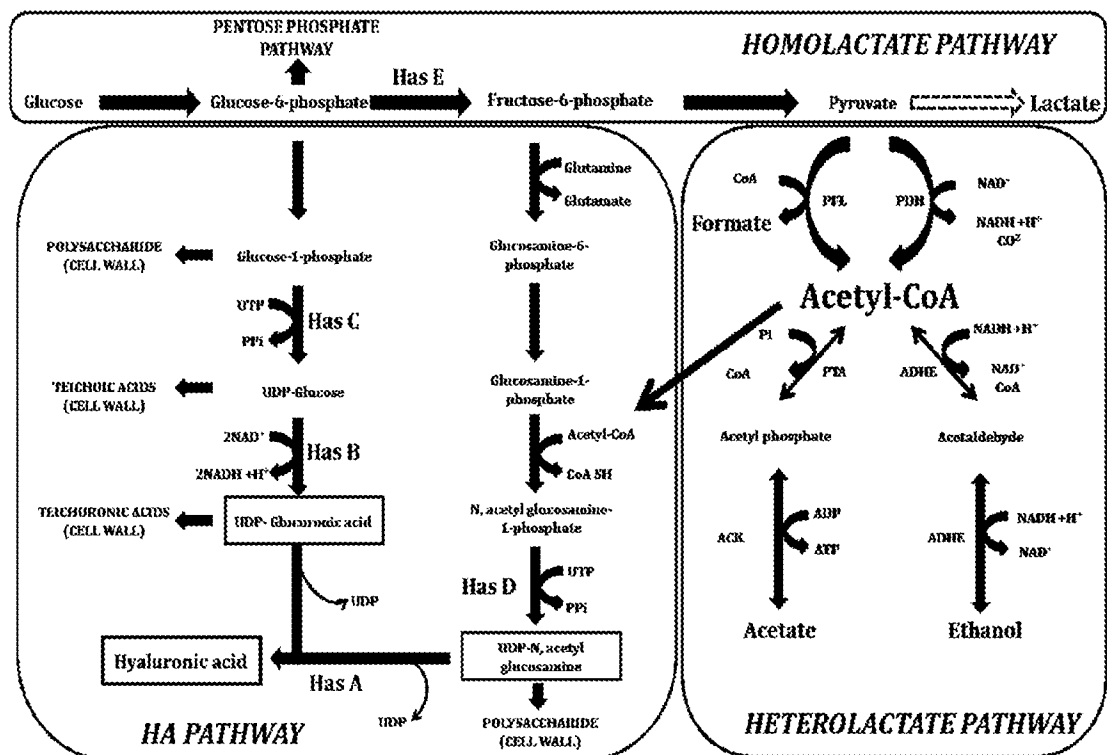


Figure 2

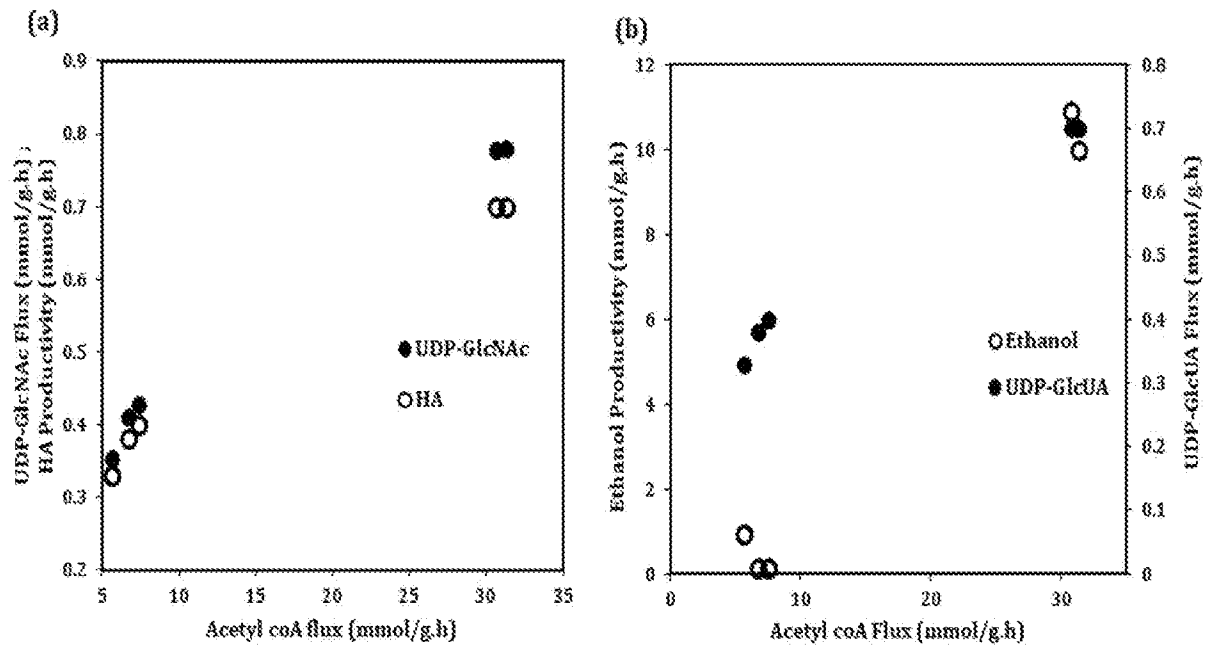


Figure 3

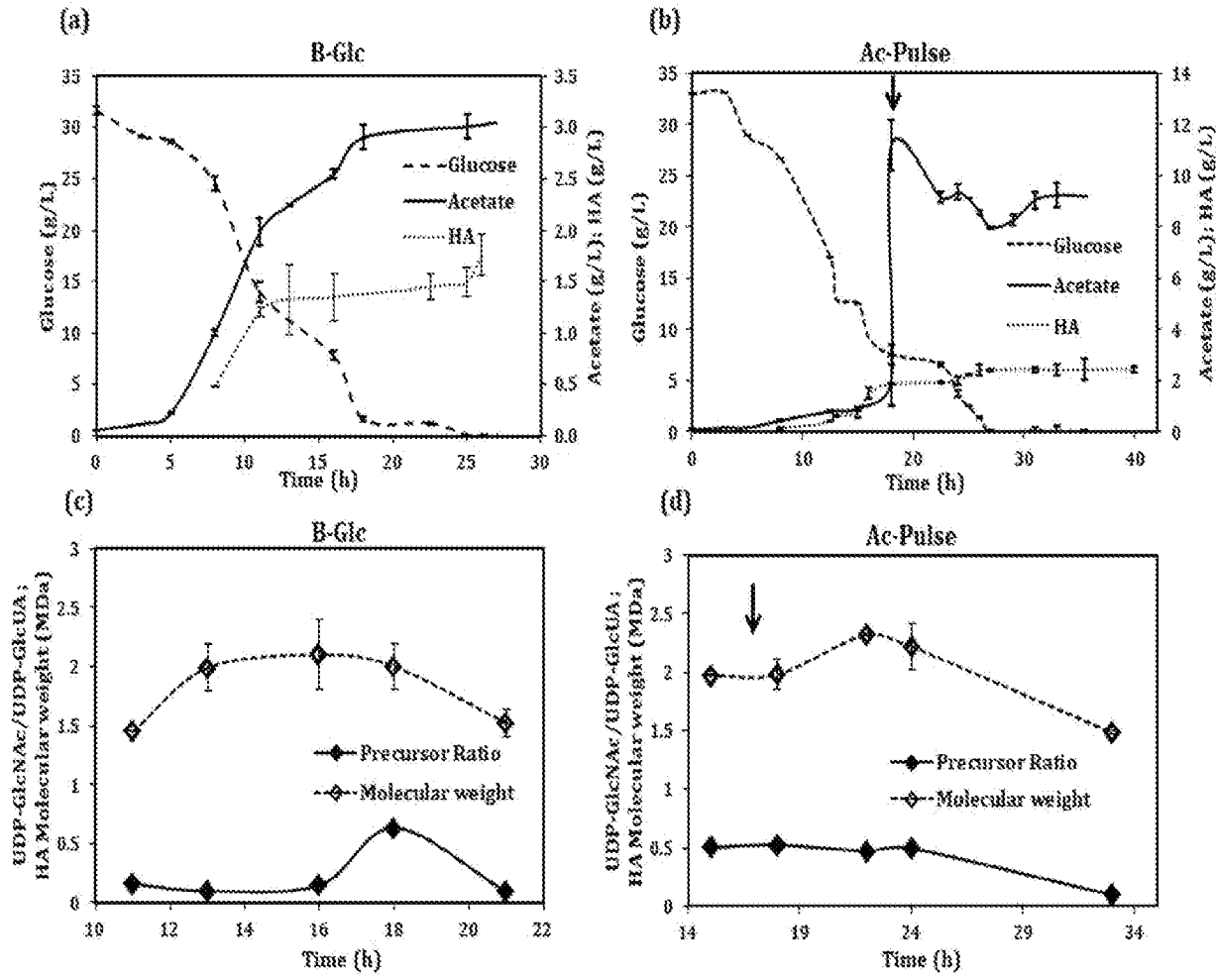


Figure 4

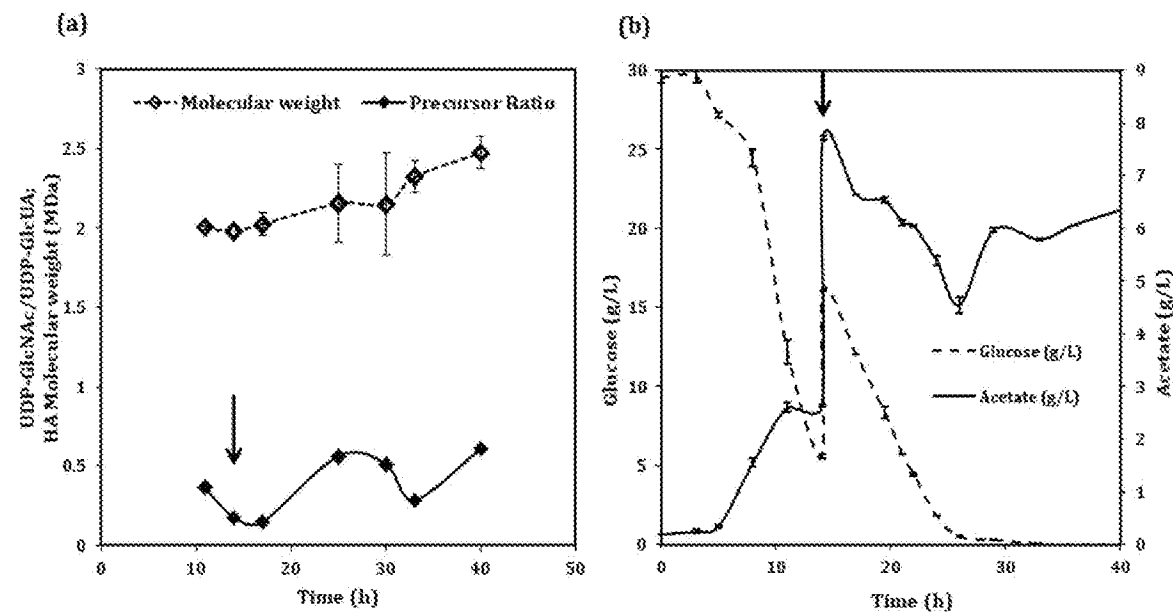


Figure 5

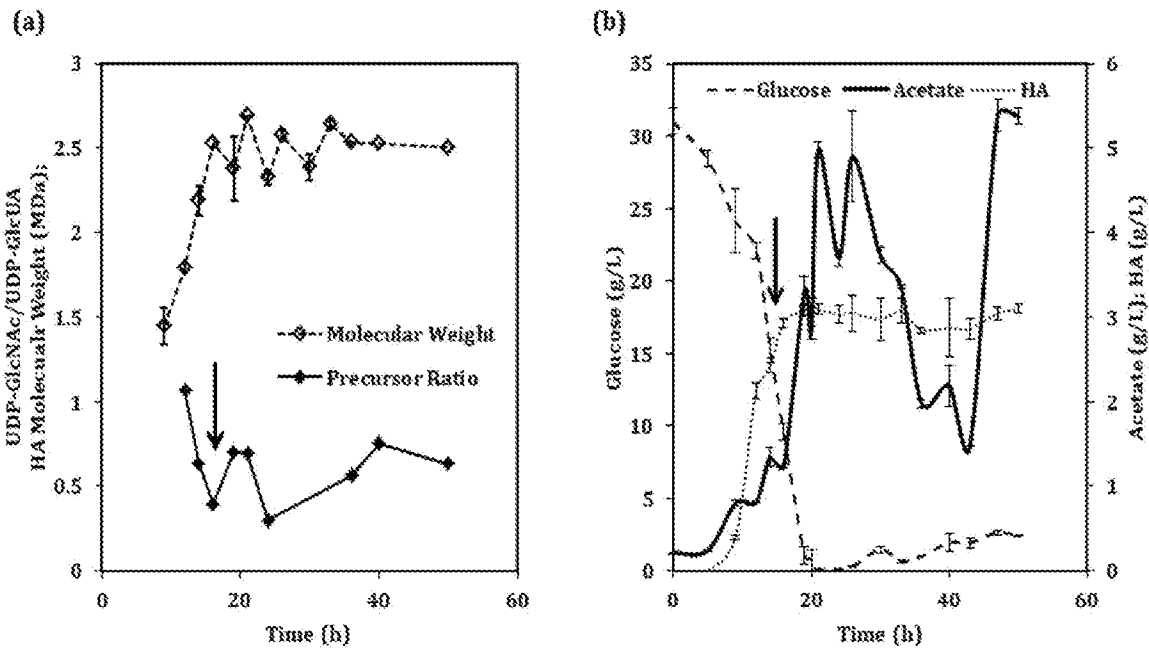


Figure 6

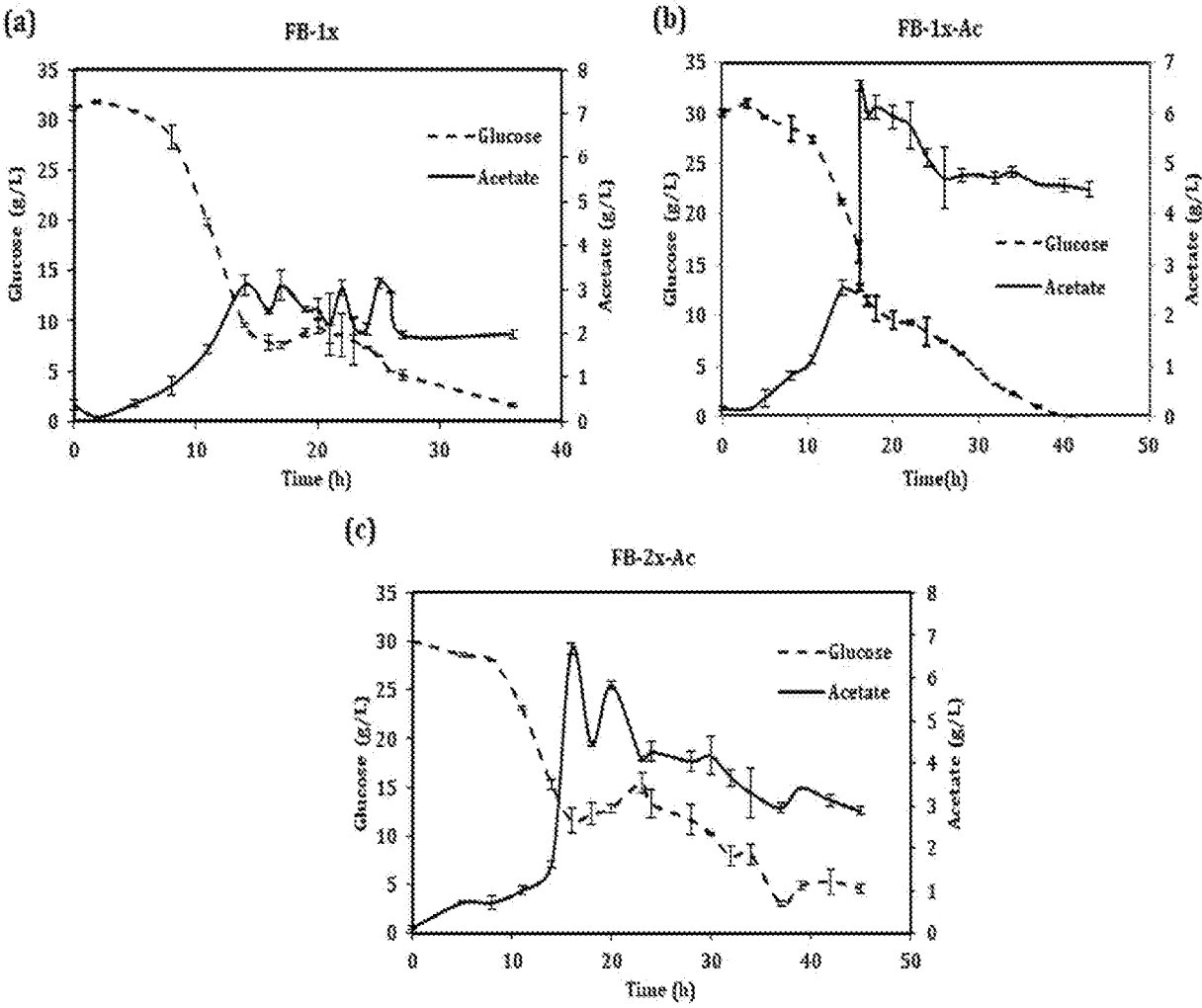


Figure 7

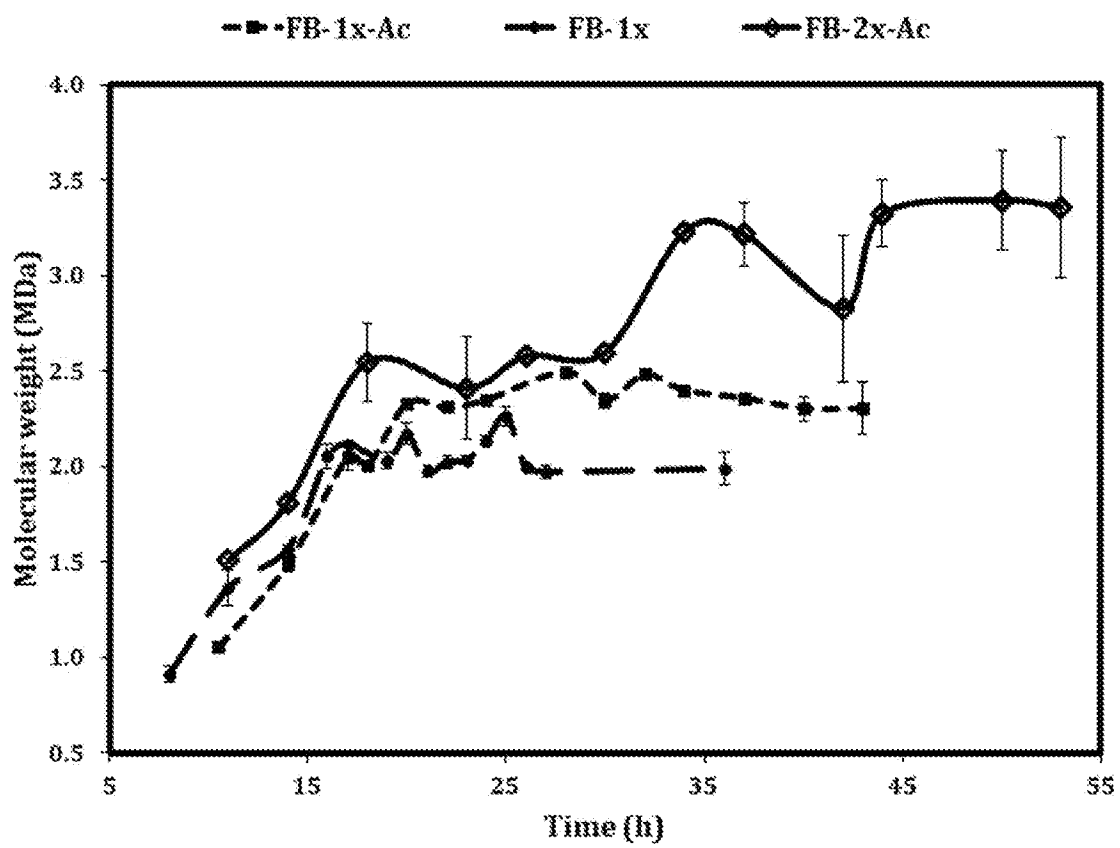


Figure 8

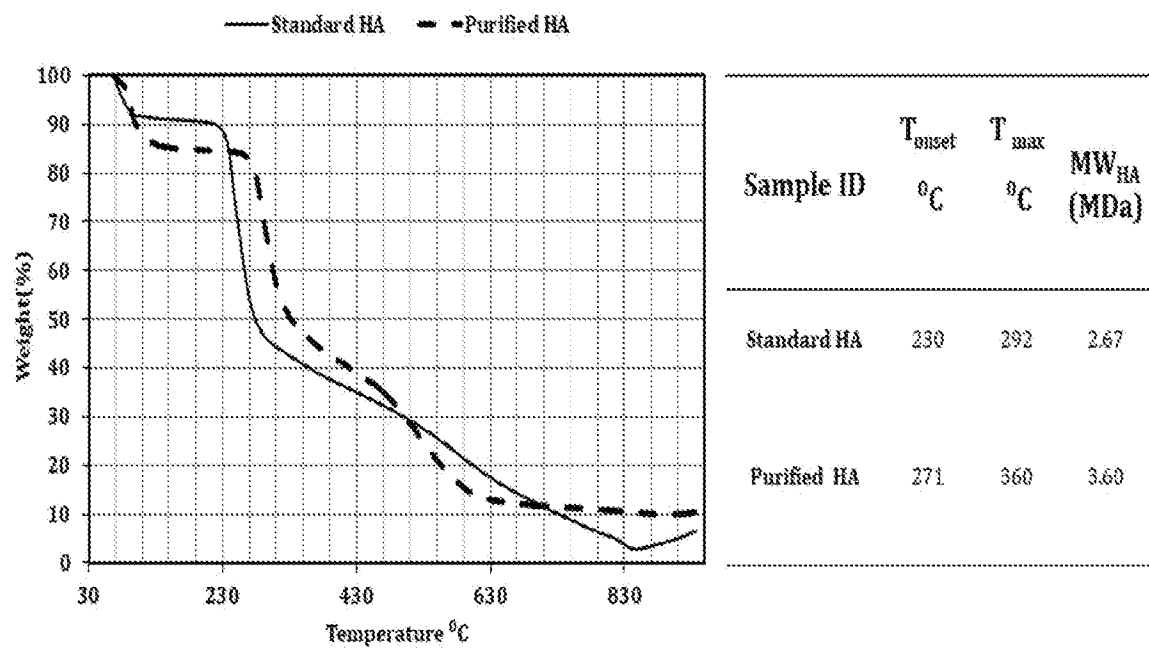


Figure 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2020/050447

A. CLASSIFICATION OF SUBJECT MATTER

C12M1/00, A61K31/728, C12R1/46 Version=2020.01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12M, A61K, C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

TotalPatent One, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Kaur, Mandeep, and Guhan Jayaraman. "Hyaluronan production and molecular weight is enhanced in pathway-engineered strains of lactate dehydrogenase-deficient <i>Lactococcus lactis</i> ." <i>Metabolic engineering communications</i> 3 (2016): 15-23. refer abstract, results and discussion, para 3.1, table 2	1-12
Y	Jeeva, Pandeewari, et al. "Production of controlled molecular weight hyaluronic acid by glucostat strategy using recombinant <i>Lactococcus lactis</i> cultures." <i>Applied microbiology and biotechnology</i> 103.11 (2019): 4363-4375. refer abstract	1-12
Y	Liu, Long, et al. "Microbial production of hyaluronic acid: current state, challenges, and perspectives." <i>Microbial cell factories</i> 10.1 (2011): 99. refer abstract, para 4	1-12



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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Date of the actual completion of the international search

10-09-2020

Date of mailing of the international search report

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