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(54) Title: PROCESS FOR PRODUCING LOW MOLECULAR WEIGHT HYALURONIC ACID OR SALT THEREOF

(57) Abstract: The present disclosure relates to process for producing low molecular weight HA or salt thereof, comprising, producing high molecular weight HA or salt thereof by fermentation; and carrying out in-situ fragmentation of said high molecular weight HA or salt thereof; to obtain low molecular weight HA or salt thereof.

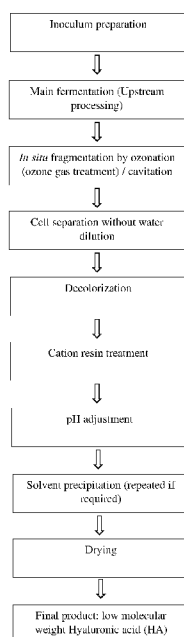


Figure 1

LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE,
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TITLE OF THE INVENTION**PROCESS FOR PRODUCING LOW MOLECULAR WEIGHT
HYALURONIC ACID OR SALT THEREOF****CROSS-REFERENCE TO RELATED APPLICATIONS AND PRIORITY**

The present application claims priority from Indian patent application number (202321089681) filed on (29/12/2023), incorporated herein by a reference.

5

TECHNICAL FIELD

The present subject matter, in general, relates to the field of process chemistry. More particularly, the present subject matter relates to process for producing low molecular weight hyaluronic acid or salt thereof, comprising, producing high molecular weight HA or salt thereof by fermentation; and carrying out in-situ
10 fragmentation of said high molecular weight HA or salt thereof; to obtain low molecular weight HA or salt thereof.

BACKGROUND

Hyaluronic acid or hyaluron or hyaluronate (HA) is essentially an unsulphated glycosaminoglycan composed of repeating disaccharide units of N-
15 acetylglucosamine (GlcNAc) and glucuronic acid (GlcUA) linked together by alternating beta-1,4 and beta-1,3 glycosidic bonds. HA is endowed with unique physiological and biological properties such as high water-holding capacity and viscoelasticity and thus occurs widely in hyaline cartilage, synovial joint fluid, and skin tissue, both dermis and epidermis, and is also suspected of playing a role in
20 numerous physiological functions, such as adhesion, development, cell motility, cancer, angiogenesis, and wound healing. Its biocompatibility accords it with unprecedented applications in the fields of medicine and cosmetics.

Extraction from rooster comb and fermentation of certain attenuated strains of group A and C *Streptococcus* are commonly exploited for producing HA or salt
25 thereof. Commercial HA extraction from rooster combs is an arduous and costly procedure, suffering from several technical limitations whereas actively sought

microbial fermentation demonstrates efficient preparation and purification at comparatively lower costs, effectuating its implementation on an industrial scale.

It is known that microbial HA production can be considerably affected by culture conditions, including temperature, pH, aeration rate, agitation speed, dissolved
5 oxygen, shear stress, and type of bioreactor. Therefore, optimization of process parameters through microbial production is invariably advisable.

The molecular weight (MW) of HA or salt thereof is the primary quality parameter that determines its suitability for various applications. High molecular weight HA or salt thereof consists of 1,500 kD and larger molecules that cannot feasibly
10 penetrate the skin barrier, and are effortlessly washed off, plummeting their real long-term effect. On the other hand, since low molecular weight HA or salt thereof easily absorbs and penetrates the deepest layers of the skin, it is exceedingly recommended for cosmetic applications.

The production of low molecular weight HA or salt thereof is commonly carried
15 out by treating the high molecular weight product with inorganic chemicals, and altering physical conditions such as, pH, temperature, agitation, selective filtration, etc. Commercial formulators also achieve low molecular weight HA or salt thereof by applying depolymerization methods involving at least one of ozone radiation, and mechanical fragmentation to the industrially finished HA.

20 In prior studies, upstream processing is followed by broth dilution step, which involves addition of water to reduce the viscosity of the harvested HA or salt thereof for down streaming. This increases the overall volume of the broth, impacting the equipment sizing and quantity of chemicals required for downstream processing. The operational cost and time are further compounded due to inclusion of auxiliary
25 operational steps such as wastewater treatment, essential for treating excess diluent. According to *Cavalcanti et al.*, the degree of purity of HA or salt thereof is a determining factor for successful industrial applications. It is known that the source as well as the purification process collaborate to determine the characteristics of the HA or salt thereof produced in terms of purity, molecular weight, yield, and cost,
30 which represents a major challenge in the field of applied research for high-quality, high yield hyaluronans. Reducing the risk of microbial contamination, especially

via microbial deactivation, has also been determinative in high quality and quantity HA production.

Conventionally, repeated purification steps in the form of solvent precipitation, adsorption, filtration, etc. are performed for efficiently removing these impurities.

5 However, these processes involve large amounts of solvents and are time-consuming.

Present market demands cosmetic grade HA or salt thereof having optimum techno commercially viable technology. In addition to this, an efficient low-cost process for recovery and purification of low molecular weight HA or salt thereof for
10 industrial scale production is also desirable. The present study aims to address these requirements by placing a commercially viable and cost-efficient technology for producing cosmetic grade HA or salt thereof.

SUMMARY

An exemplary embodiment of the present disclosure relates to a process for
15 producing low molecular weight hyaluronic acid (HA) or salt thereof, comprising, producing high molecular weight HA or salt thereof by fermentation; and carrying out *in-situ* fragmentation of said high molecular weight HA or salt thereof; to obtain low molecular weight HA or salt thereof.

A particular embodiment of the present disclosure relates to a process for producing
20 low molecular weight HA or salt thereof, wherein the *in-situ* fragmentation is carried out by at least one of ozonation or cavitation.

This summary is not intended to identify all the essential features of the claimed subject matter, nor is it intended to be used in determining or limiting the scope of the claimed subject matter.

BRIEF DESCRIPTION OF DRAWINGS

25 The detailed description of drawings is outlined with reference to the accompanying figures. In the figures, the left-most digit (s) of a reference number identifies the Figure in which the reference number first appears. The same numbers are used throughout the drawings to refer like features and components.

Fig. 1 illustrates a process flow diagram (PFD) for producing low molecular weight HA.

Fig. 2 demonstrates gel electrophoresis run wherein, low molecular weight ladder has 5 HA bands (32, 74, 198, 400 and 480 kDa); high molecular weight ladder has 5 HA bands (480, 547, 762, 932, and 1272 kDa). Size of various Sigma HA standards are as indicated on the gel. Purified HA samples (1 to 6) by three different processes migrate at high, medium, and low molecular range as indicated. Samples 1 & 2: conventional process for producing HA samples; Samples 3 & 4: *in-situ* ozone gas treated samples; Sample 5: *in-situ* cavitation treated samples; Sample 6: conventional process for producing HA samples.

Fig. 3 demonstrates GPC result showing variation in the molecular weight of HA with respect to different time intervals during *in-situ* ozonation/ozone treatment.

DETAILED DESCRIPTION

Reference throughout the specification to “various embodiments,” “some embodiments,” “one embodiment,” “alternate embodiment”, or “an embodiment” means that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment. Thus, appearances of the phrases “in various embodiments,” “in some embodiments,” “in one embodiment,” or “in an embodiment”, “in an alternate embodiment”, or “in a related embodiment” in places throughout the specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures or characteristics may be combined in any suitable manner in one or more embodiments.

Reference throughout the specification to “components”, “component”, “features”, or “feature” means a constituent or group of constituents embodying the process.

Before the present process is described, it is to be understood that this disclosure is not limited to the particular process as described, as there can be multiple possible embodiments which are not expressly illustrated in the present disclosure but may still be practicable within the scope of the present disclosure.

Also, the technical solutions offered by the present disclosure are clearly and completely described below. Examples in which specific conditions may not have

been specified, have been conducted under conventional conditions or in a manner recommended by the manufacturer.

Instant disclosure relates to process chemistry; and particularly relates to a process for producing low molecular weight HA or salt thereof.

- 5 An exemplary embodiment of the instant disclosure relates to a process for producing low molecular weight HA or salt thereof, comprising, producing high molecular weight HA or salt thereof by fermentation; and carrying out *in-situ* fragmentation of said high molecular weight HA or salt thereof; to obtain low molecular weight HA or salt thereof.

- 10 For the purpose of the instant disclosure, “hyaluronic acid” or “HA” or “hyaluronate” denote hyaluronic acid.

An aspect relates to a process for producing low molecular weight HA or salt thereof.

- 15 An embodiment relates to a process for producing HA; and preferably, low molecular weight HA.

Another embodiment relates to a process for producing salt of HA; and preferably, salt of low molecular weight HA.

- In a related embodiment, the salt of hyaluronic acid (HA salt) is at least one of sodium hyaluronate and potassium hyaluronate; and preferably, sodium hyaluronate.
- 20

A preferred embodiment of the instant disclosure relates to a process for producing low molecular weight HA and salt thereof.

- Another aspect of the instant disclosure relates to producing high molecular weight HA or salt thereof; particularly, by fermentation.
- 25

A related embodiment relates to isolating HA producing strain; preferably, for producing high molecular weight HA or salt thereof; particularly, by fermentation.

In an embodiment HA producing strain is one or more bacterial or yeast HA producing strain; and preferably, one or more bacterial HA producing strain.

For the purpose of instant disclosure, the terms “strain”, “strains”, “microbe(s)”, “organism(s)” pertain to a “microorganism(s)”, as is perceivable to a person skilled in the art and/or as is fundamentally defined.

5 Related embodiment relates to isolating one or more HA producing strain using an isolation medium.

For the purpose of instant disclosure, the terms “media”, “medium”, “culture solution”, “fermentation solution” “fermentation broth”, “base medium”, “fermentation medium”, “enrichment medium”, “inoculum medium”, “pre-fermentation medium”, “isolation media/medium”, or “broth” pertain to at least one
10 of solid, liquid, gaseous, semi-solid, semi-liquid, synthetic, semi-synthetic, or non-synthetic, culture medium which is essentially composed of basic elements and/or growth factors for growing, incubating, or optimizing one or more organism.

In a preferred embodiment, Brain heart infusion (BHI) medium/broth is the isolation medium.

15 Process parameters, as are fundamentally perceivable to a person skilled in the art, are adjusted, as per the requirements.

In yet another embodiment, the isolation medium is screened for positive result; and particularly, for HA production.

20 Another embodiment of the instant disclosure relates to identifying one or more HA producing strain; preferably, using conventionally known identification methods; and particularly, using conventionally known molecular identification methods such as gene sequencing.

25 In a related embodiment relates to inoculum preparation; preferably, using an inoculum medium; more preferably, using Hi-veg hydrolysate medium; and particularly, in a reactor.

For the purpose of this disclosure and as is perceivable to a person skilled in the art, the term “reactor” or “fermenter” pertains to generator, bioreactor, vessel, jar, tank,
30 or any manufactured device or system that supports a biologically active environment. In a further embodiment, the “reactor” or “fermenter” is rectangular,

cylindrical, or circular shaped; and preferably, cylindrical shaped. Furthermore, in a related embodiment, the “reactor” or “fermenter” is horizontally, diagonally, or vertically angled. In another embodiment, the “reactor” or “fermenter” is a small, medium, or large capacity “reactor” or “fermenter”. Furthermore, in a related
5 embodiment, volume of “reactor” or “fermenter” is any volume from 1L to several thousands of L, as per requirements.

In an embodiment, size of inoculum ranges from 0.1 % to 5%; and preferably ranges from 0.1% to 3%.

For the purpose of instant disclosure, and as is fundamentally perceivable to a
10 person skilled in the art, the term “inoculum” refers to population, concentration, or volume of strains or microbes introduced in the medium.

In another embodiment, temperature of incubation is $\leq 55^{\circ}\text{C}$; and preferably, $\leq 45^{\circ}\text{C}$. In a further embodiment, duration of incubation is ≤ 40 h; and preferably, ≤ 30 h. In a further embodiment, the mixing speed is ≤ 250 rpm; and preferably, ≤ 200
15 rpm.

In yet another related embodiment, the fermentation is carried out by *Streptococcus* spp.; preferably, by at least one of *Streptococcus equi*, or *Streptococcus zooepidemicus*; and particularly, by *Streptococcus zooepidemicus*.

20 In an exemplary embodiment, the fermentation is carried out by *Streptococcus zooepidemicus* (MTCC 25714).

A further related embodiment relates to main fermentation (upstream processing).

A related embodiment relates to optimizing process parameters for producing HA or salt thereof.

25 In an embodiment, pH is optimized to ≥ 5 ; and preferably to ≥ 6 .

In another embodiment, aeration is optimized to ≤ 5 vvm; and preferably, to ≤ 2.5 vvm.

In a further embodiment, temperature is optimized to $\leq 55^{\circ}\text{C}$; and particularly, $\leq 45^{\circ}\text{C}$.

30 In yet another further embodiment, mixing speed is optimized to ≤ 4 m/s; and preferably, ≤ 3 m/s.

In a related embodiment, size of inoculum ranges from 0.1 % to 10%; and preferably ranges from 3% to 7%.

5 In a particular embodiment, HA producing strain is adapted; preferably using isolation medium; and particularly using optimized process parameters described above.

For the purpose of instant disclosure, and as is perceivable to a person skilled in the art, the term “adapted” pertains to performing/carrying out gradual modification(s) of microorganisms in stressful environment to enhance their tolerance. During
10 adaptation, microorganisms use different mechanisms to enhance non-preferred substrate utilization and stress tolerance, thereby improving their ability to adapt for growth and survival.

In an embodiment, the fermentation is carried out in fermentation media/medium; and preferably, comprising at least one of dextrose monohydrate, yeast extract,
15 sodium chloride, magnesium sulfate, di-potassium phosphate, and soya oil.

In a particular embodiment, the concentration/quantity of dextrose monohydrate ranges from 25 g/L to 60 g/L; and preferably, from 35 g/L to 50 g/L. The concentration/quantity of yeast extract ranges from 10 g/L to 50 g/L; and preferably, from 20 g/L to 40 g/L. The concentration/quantity of sodium chloride ranges from
20 0.1 to 10 g/L; and preferably, from 0.1 to 5 g/L. The concentration/quantity of magnesium sulfate ranges from 0.1 g/L to 7 g/L; and preferably, from 0.1 g/L to 5 g/L. The concentration/quantity of di-potassium sulfate ranges from 0.1 to 10 g/L; and preferably, from 0.1 to 7 g/L. The concentration/quantity of soya oil ranges from 0.1 to 10 g/L; and preferably, from 0.1 to 5 g/L.

25 In a preferred embodiment, the fermentation is carried out to obtain fermentation broth.

In a related embodiment, pH, sugar concentration, optical density, and viscosity of the fermentation broth is monitored; preferably, every 2 h; and particularly, every
1 h.

30 As described earlier, an aspect of instant disclosure relates to producing high molecular weight HA or salt thereof by fermentation.

In a related embodiment, molecular weight of the high molecular weight HA or salt thereof is ≥ 2000 kDa; and preferably, ≥ 1800 kDa.

In a preferred embodiment, the fermentation is carried out till fermentation viscosity is ≥ 2200 cp; and preferably, ≥ 2000 cp.

5

Yet another aspect of instant disclosure relates to carrying out fragmentation of the high molecular weight HA or salt thereof; particularly to obtain low molecular weight HA or salt thereof.

An embodiment relates to carrying out *in-situ* or *ex-situ* fragmentation of the high molecular weight HA or salt thereof; and particularly, *in-situ* fragmentation.

For the purpose of instant disclosure and as is perceivable to a person skilled in the art, the term “fragmentation” or “fragmentized” pertains to a process in which molecular ions are broken into smaller their smaller counterparts such as, ions, radicals, and/or neutral molecules. Furthermore, in “*in-situ*” fragmentation, fermentation broth is implemented (for fragmentation). Conversely, in the conventionally known “*ex-situ*” fragmentation, final product, or HA, as is described in the instant case, is implemented (for fragmentation).

In a preferred embodiment, the *in-situ* fragmentation is carried out after the fermentation broth achieves viscosity of ≥ 2200 cp; and preferably, ≥ 2000 cp.

20

In an embodiment, the *in-situ* fragmentation is carried out by ozonation or ozone treatment; and preferably, by ozone gas treatment.

For the purpose of instant disclosure and as is perceivable to a person skilled in the art, the term “ozonation” or “ozone gas treatment” or “ozone treatment” refers to an advanced oxidation process (AOP) using ozone (O_3).

In a related embodiment, ozonation is carried out at 2 g/h to 20 g/h; and preferably, 5 g/h to 15 g/h.

Alternatively, in a preferred embodiment, ozonation is carried out at 2 lpm to 15 lpm; and particularly, at 6 lpm to 10 lpm.

In another related embodiment, ozonation is carried out for 1 to 12 h, preferably for 1 to 10 h; and particularly, at 30°C to 70°C, preferably, at 35°C to 65°C.

Alternatively, the *in-situ* fragmentation is carried out by cavitation; preferably, with or without addition of hydrogen peroxide (H₂O₂); and particularly, with addition of H₂O₂.

- 5 For purpose of instant disclosure and as is perceivable to a person skilled in the art, the term “cavitation” pertains to a process whereby pressure variations in a liquid can in a short period of time cause countless small cavities to form and then implode.

In a related embodiment, the cavitation is carried out at 2 m³/h to 52 m³/h; preferably, with 2 kg/m³ to 12 kg/m³ pressure; and particularly, for 1 h to 5 h, preferably, for 1. h to 3. h.

In another related embodiment, the cavitation is carried out at 25°C to 50°C; and preferably, at 30°C to 45°C.

- 15 In a preferred embodiment, the *in-situ* fragmentation reduces the fermentation viscosity to ≤ 100 cp; and preferably, to ≤ 50 cp.

In yet another preferred embodiment, the *in-situ* fragmentation reduces microbial contamination; and particularly, deactivates infectious organisms.

- For the purpose of instant application, and as is perceivable to a person skilled in the art, the term “infectious organisms” relate to conventionally known microbial agents associated with human diseases that pose moderate health hazard.

In an embodiment, cell separation step is carried out after *in-situ* fragmentation; preferably, using centrifugation; and particularly, without any requirement for addition of water/ fermentation broth dilution.

- 25 In a related embodiment, centrifugation is carried out at 10000 rpm to 20000 rpm; and preferably, at 12000 rpm to 18000 rpm. Process parameters, as are fundamentally perceivable to a person skilled in the art, are also adjusted, as per the requirements.

- In a preferred embodiment, supernatant comprising HA or salt thereof is obtained after the cell separation.

In another embodiment, the supernatant comprising HA or salt thereof is decolorized; preferably by using an adsorbent selected from activated carbon, activated charcoal, aluminium silicate, silica gel, magnesium silicate or combinations thereof known to a skilled person; and particularly, using activated
5 carbon. It is known that an adsorbent is conventionally used for removing/separating undesirable components from a solution. In a preferred embodiment, decolorized supernatant comprising HA or salt thereof is obtained after the decolorization / decolorization step.

10 In yet another embodiment, the decolorized supernatant comprising HA or salt thereof is purified; preferably using at least one of cation resin treatment, membrane filtration, electrodialysis, and other conventionally known methods of demineralization/purification; and particularly, using cation resin treatment. It is known that cation resin treatment helps in de-mineralizing, and de-alkalizing. They
15 have high capacity strongly acidic cationic exchanger, containing sulphonic acid groups, and are based on crosslinked polystyrene with a gel structure, that have a higher degree of cross linkage to facilitate feasible removal of impurities.

In a preferred embodiment, decolorized and purified supernatant comprising HA or salt thereof is obtained after cation resin treatment.

20 In a further embodiment, pH of the decolorized and purified supernatant comprising HA or salt thereof is adjusted; preferably to ≥ 3 pH. For this, at least one of sodium acetate, or potassium acetate is implemented; and particularly, sodium acetate is implemented.

In yet another further embodiment, after adjusting pH, the decolorized and purified
25 supernatant comprising HA or salt thereof is subjected to solvent precipitation.

It is fundamentally known that cosmetic, personal care, and pharmaceutical applications requisite solvents that are safe to be used in tropical applications. Thus, high purity solvents that are safe for consumption or contact with the human body, such as food grade solvents; and preferably, class 3 food grade solvents are
30 recommended.

In an embodiment, the solvent precipitation is carried out by at least one of acetone, hexane, ethyl acetate, ethanol, n-propanol, isopropanol, or mixtures thereof.

In a preferred embodiment, the solvent demonstrates enhanced yield, and selectivity; preferably, lower residual value; and particularly, cost-effectiveness and availability.

Acetone demonstrates better yield and separation of HA or salt thereof from impurities to provide purer final product, as well as lower residual level of about $\leq$ 0.1%. Further, in addition to cost-effectiveness, and availability, acetone provides better control and reproducibility in the precipitation process, which facilitates process optimization and ensures consistent product quality. This positively effectuates easier scaling up for reliable industrial HA or salt thereof production.

Accordingly, in a preferred embodiment, solvent precipitation is carried out by acetone; particularly, in a ratio of 1:1 to 1:5 and following the precipitation, the solvent may be recovered and reused. In a related embodiment, the precipitate is washed with solvent; preferably to obtain final product or HA.

In another related embodiment, final product (HA or salt thereof) is dried; particularly, to analyze as per industry standards; preferably, as recommended in European Pharmacopoeia - Supplement 2001.

In an exemplary embodiment, after strain isolation, inoculum preparation, and main fermentation as described in foregoing paragraphs, ozone gas is introduced in the fermentation broth having viscosity of $\geq$ 2000 cp at about 5 g/h to 15 g/h (2 lpm to 15 lpm) flow rate for about 60-250 mins (1-5 h), at about 35°C -60°C under mixing conditions (100 rpm - 800 rpm). The fermentation broth is also screened for infectious organisms using microscopy (cell lysis %), and TVC for CFU/ml on BHI media after the ozone gas treatment. Once the fermentation broth viscosity reduces to about $\leq$ 50 cp (after about 1-5 h of ozone gas treatment), cell separation is initiated. Separated cell biomass is sent to incineration for disposable purpose, and supernatant is decolorized using granular activated carbon. The decolorized supernatant comprising HA or salt thereof is then passed through cation resin at a

flow rate of about 50-180 mL/min to demineralize and de-alkalize, and especially to remove inorganic impurities. After the cation resin treatment, pH of the decolorized and purified supernatant comprising HA or salt thereof is adjusted to ≥ 3 . The decolorized and purified supernatant comprising HA or salt thereof is subjected to solvent precipitation using acetone in about 1-5:5-1 ratio. Precipitate is further washed with acetone to remove water from the product and dried overnight at about 30-60 °C overnight to get a final product (HA or salt thereof).

In another exemplary embodiment, strain isolation followed by inoculum preparation, and main fermentation as described in foregoing paragraphs, is subjected to cavitation (with or without addition of hydrogen peroxide (H_2O_2)) of fermentation broth having viscosity of ≥ 2000 cp at about 1 m³/h to 7 m³/h with about 1 kg/m³ to 15 kg/m³ pressure, for about 1-5 h at about 37°C. Once the fermentation broth viscosity is reduced to about ≤ 50 Cp, cell separation, decolorization, cation resin treatment, and solvent precipitation steps are performed as described in foregoing paragraphs to obtain a final product (HA).

Moreover, following the precipitation, the solvent may be recovered and reused.

In a particular embodiment, the final product is low molecular weight HA or salt thereof. Alternatively, the final product is super low molecular weight HA or salt thereof.

In an embodiment, molecular weight of the low molecular weight HA or salt thereof is ≤ 1000 kDa; and preferably, is ≤ 800 kDa. In a preferred embodiment, purity of the low molecular weight HA or salt thereof is $\geq 70\%$; and particularly, $\geq 80\%$.

In another preferred embodiment, loss on drying of the low molecular weight HA or salt thereof is $\leq 30\%$; and particularly, $\leq 20\%$.

In a further preferred embodiment, recovery of the low molecular weight HA or salt thereof is $\geq 75\%$; and particularly, $\geq 80\%$. In yet another further preferred embodiment, bacterial count of the low molecular weight HA or salt thereof is ≤ 50 CFU/g; and particularly, ≤ 20 CFU/g.

In a preferred embodiment, the low molecular weight HA or salt thereof is applicable in cosmetics.

Various modifications to the embodiment will be readily apparent to those skilled in the art and the generic principles herein may be applied to other embodiments. However, one of ordinary skill in the art will readily recognize that the present disclosure is not intended to be limited to the embodiments illustrated but is to be accorded the widest scope consistent with the principles and features described herein.

The foregoing description shall be interpreted as illustrative and not in any limiting sense. A person of ordinary skill in the art would understand that certain modifications could come within the scope of this disclosure. The features and properties of the present disclosure are described in further detail below with reference to examples.

Example 1

Isolation of HA producing strain(s).

Soil sample collected from a barn/stable at Hadapsar, Pune (The NBA ABS filing number is INBA1202305181) was serially diluted using sterile water and then spread on commercially available BHI medium (about 3.7%) comprising about 20% of calf brain infusion, about 25% of beef heart infusion, about 1.0% of protease peptone, about 0.5% of NaCl, about 0.25% of Na₂HPO₄, and about 0.2% of dextrose. Isolated strain showing positive result was analysed using NCBI Blastn analysis (molecular identification) and showed $\geq 99\%$ homology to *Streptococcus zooepidemicus* (MTCC 25714).

Example 2

Inoculum preparation

Freshly grown colonies of *Streptococcus zooepidemicus* (11C10) from **Example 1** were inoculated in about 50 ml of Hi-veg hydrolysate medium (*see Table 1*) and incubated at about 37 °C for about 20 h, at about 150 rpm.

Table 1: Composition of Hi-veg hydrolysate medium (seed ingredients) (for 1000 ml)

Ingredient	Quantity (g/L)	Required 0.3 L (g)
Hi-veg hydrolysate No. 1 (<i>HiMedia</i>)	30	9
Sodium chloride (NaCl)	5	1.5
Dipotassium phosphate (K ₂ HPO ₄)	2.5	0.75
Glucose	10	3
Water	1	300
pH	6.8 ± 0.1	6.9

Then about 800 ml of hi-veg hydrolysate medium (1% v/v of inoculum) was transferred to about 2L - 2.125L volume reactor (comprising of hi-veg hydrolysate medium) and incubated at about 37°C for about 20 h, at about 150 rpm.

Example 3

5 Optimization of parameters for Main fermentation (upstream processing)

First process parameters such as pH, aeration, mixing, and temperature were optimized through optical density (OD) (*see Tables 2-5*) by adding about 2.125 ml of inoculum prepared as per **Example 2** in about 42.5 ml of fermentation media (*see Table 6*) subjected to different values of the said parameters.

10 Table 2: pH variation with respect to optical density

Sr. No	pH	OD
1.	6.7	11.5-14
2.	7	14-18
3.	7.3	12-15.4

Table 3: Aeration study with respect to optical density

Sr. No	Air (vvm)	OD	Viscosity	Remark
1.	0.8	12.5	1410	Comparatively lower OD
2.	1	14-18	2230	Optimum growth
3.	1.2	14-18	2190	Foaming observed

Table 4: Temperature variation data with respect to OD

Sr. No	Temperature (°C)	OD
1.	34	12-14
2.	37	14-18
3.	40	12-15

15 .

Table 5: Mixing study with respect to optical density.

Sr. No	Mixing (Tip speed, m/s)	OD	Viscosity	Remark
1.	1.5	12-14	1237	Low OD

2.	2	14-16	2150	Optimum growth
3.	2.5	13-15	2170	Foaming observed

As per **Tables 2-5**, the optimum pH, aeration, temperature, and mixing speed were identified to be 7, 1 vvm, 37°C, and 2 m/s, respectively. *Streptococcus zooepidemicus* (11C10) from **Example 1** was adapted for high titre of HA or salt thereof using BHI medium/broth and optimized process parameters established here.

Example 4

Main fermentation (upstream processing)

About 5% of the adapted inoculum (about 2.125 L) was added in the fermentation medium and fermented at about 6.9 pH, 1 vvm aeration, 1800 Kcal.kg of sugar heat of reaction, 3 psi air pressure, 190 rpm, 37°C for about 16-24 h.

Table 6: Composition of fermentation medium

Sr. No.	Component	Value	For 42.5 L
1	Dextrose monohydrate	44 g/L	--
2	Yeast extract	30 g/L	1275
3	Sodium chloride	2 g/L	85
4	Magnesium sulfate	1.5 g/L	63.75
5	Di-potassium phosphate	2.5 g/L	106.25
6	Soya oil	1 g/L	42.5
7	Final make up with water	--	35.4
Sterilized at 121°C for 20 min			

The makeup volume was about 35.4 L at the time of sterilization. About 44 g/L (1870 g) of glucose was taken on basis of about 42.5 L initial volume of fermentation and dissolved in water. Sugar solutions were separately sterilized and added before inoculation. Throughout the fermentation, pH was maintained at about 6.9 using about 30 % sterilized NaOH solution. After 8 hours of fermentation, once the sugar concentration reached 1% w/w, sterilized 66 % dextrose (about 6.25 L) was added to the fermenter through feeding bottle as per requirement to maintain sugar concentration at 0.5 % w/v.

The pH, sugar concentration, optical density, and viscosity of the fermentation broth were monitored every 1 h. A total of about 50 L of broth was harvested.

Table 8 illustrates viscosity monitored during fermentation.

[Table 8]

Sr. No.	Fermentation retention time (h.)	Viscosity (Cp)
1	10	850
2	12	1310
3	14	1734
4	16	1970
5	18	2258

Once the fermentation broth achieved viscosity of ≥ 2000 cp after about 18 h, *in-situ* fragmentation was initiated.

Example 5

***In-situ* fragmentation using ozonation (ozone gas treatment)**

Ozone gas was introduced in the fermentation broth of **Example 4** having viscosity of ≥ 2000 cp at about 10 g/h (at about 6 lpm to 10 lpm) flow rate for about 180 min (3 h), at about 50°C in mixing conditions (500 rpm). **Table 9** illustrates the effect of ozone gas treatment on the molecular weight of HA or salt thereof and the viscosity of the fermentation broth.

[Table 9]

Ozone treatment time (Hrs)	Viscosity (Cp)	Molecular weight (kDa)
0	2100	1500-2500
1	1340	1500-2000
2	470	1200-1600
3	≤ 50	600-800
4	≤ 50	400-600
5	≤ 50	200-400
6	≤ 50	50-200

Example 6

***In-situ* fragmentation using cavitation**

In-situ cavitation (with or without addition of hydrogen peroxide (H_2O_2)) was carried out in the fermentation broth of **Example 4** having viscosity of ≥ 2000 cp at about 2 m³/h to 52 m³/h with about 2 kg/m³ to 12 kg/m³ pressure, for about 2 h. The temperature was maintained in the reactor at 37°C. **Table 10** illustrates the effect of cavitation treatment on the viscosity of the fermentation broth.

[Table 10]

Time	0	0.5	1	1.5	2
Viscosity (cp) without addition of H ₂ O ₂	2365	1677	1241	963	678
Viscosity (cp) with addition of H ₂ O ₂	2295	1230	283	≤50	≤50

Example 7

Process for producing HA, wherein *in-situ* fragmentation is carried out by ozonation (see Fig. 1)

Isolation, inoculum preparation, and main fermentation was carried out as described in **Examples 1-4**. After the fermentation broth achieved viscosity of ≥ 2000 cp after about 18 h of main fermentation, *in-situ* fragmentation was performed using ozonation (ozone gas treatment) as described in **Example 5**. The fermentation broth was also screened for infectious organisms using microscopy (cell lysis %), and TVC for CFU/ml on BHI media after the ozone gas treatment. The obtained TVC count was ≤ 1000 CFU/ml.

Cell separation step

Once the viscosity of the fermentation broth is reduced to about ≤ 50 cp after about 3 h of ozone gas treatment (see **Table 9**) at about 40°C, and tip speed of 1.8 m/s, cell separation was initiated. A total of about 48 L of fermentation broth was subjected to centrifugation. Handling loss was about 2 L during the ozone gas treatment. The cell separation step was carried out without water dilution using batch centrifugation (bowl centrifuge) at about 14000 rpm, and about 37°C for about 2 h. Separated cell biomass sent to incineration for disposable purpose. A total of about 45 L of supernatant comprising HA or salt thereof was collected with about 3 L hold up volume.

Decolorization step:

Color removal or decolorization by granular activated carbon was carried out by passing the supernatant through the charcoal bed at a flow rate of about 120 mL/min. Six bed volumes of the supernatant were passed through the charcoal column. A total of about 52 L of decolorized supernatant comprising HA or salt

thereof was collected including about 1 BV (Bed Volume) of water to remove product from the column.

Cation resin treatment:

After charcoal treatment, the decolorized supernatant comprising HA or salt thereof
5 was passed through cation resin at a flow rate of about 120 mL/min (around 1 BV/h) to demineralize and de-alkalize, and especially to remove inorganic impurities such as, iron, zinc, copper, nickel, chromium etc. The cation resin (having a high capacity strongly acidic cation exchanger containing sulphonic acid groups. It is based on crosslinked polystyrene with a gel structure and has a higher degree of cross
10 linkage.) volume was about 5L. The elution water for cation was about 5-7 L.

For this experiment, a total of ten bed volumes of the decolorized supernatant comprising HA or salt thereof was passed through the resin. A total of about 57 L of decolorized and purified supernatant comprising HA or salt thereof was collected including about 1 BV of water from the resin.

15 After the cation resin treatment, pH of the decolorized and purified supernatant comprising HA or salt thereof was adjusted to about 4.7 by passing sodium acetate through cation resin. A total of about 3 kg of sodium acetate was required to adjust the pH.

Solvent precipitation:

20 The decolorized and purified supernatant comprising HA or salt thereof was subjected to solvent precipitation using acetone in about 1:2 ratio. A total of about 120 L of acetone was added at about 40 L/hr flow rate. Precipitate was allowed to settle for about half an hour and separated from the water-acetone mixture. Precipitate was further washed with acetone to remove water from the product and
25 dried overnight at about 35 °C overnight to get the final product (HA), that was analyzed/characterized. The solvent was recovered and reused.

Final product characterization/analysis

Table 11, and Figs. 2, and 3 illustrate the effect of ozonation on molecular weight (kDa) of HA.

30 **[Table 11]**

Batch Name	Molecular Weight (kDa)
High molecular weight before ozone treatment (kDa)	
1	1883
2	3221
3	2240
Low molecular weight after ozone treatment (kDa)	
1	100
2	450
3	176

As per **Table 11**, reduction in molecular weight of HA or salt thereof was achieved by way of *in-situ* fragmentation carried out using ozonation, resulting in the production of low molecular weight and/or super low molecular weight HA and/or salt thereof.

- 5 Intrinsic viscosity, purity, and loss of drying of the final product was analysed as recommended in pages 1415-1418 of EUROPEAN PHARMACOPEDIA-SUPPLEMENT 2001.

Table 12 summarizes loss of drying, purity, and intrinsic viscosity for HA or salt thereof production at 100L scale.

10 [Table 12]

Batch name	Loss of drying (LOD) % w/w	Purity (% assay)	Intrinsic Viscosity at 250C (kg/m ³)
1	15.7	82.33	1.0638
2	11.25	93.5	1.0174
3	16.26	82.52	1.02
4	12.56	88.43	1.0125
5	7.99	82.23	1.02

- As per **Tables 11-12, and Figs. 2-3**, the purity, intrinsic viscosity, molecular weight, and loss on drying of the final product was found to be about 92.7 %w/w, 1.05 kg/m³, 172 kDa and 14 % respectively. Further, the productivity and yield were found to be 0.25 g of HA/L of broth/h, and 0.037 g/g for about 4.5 g/L titre respectively. The % of recovery was $\geq 80\%$, and the bacterial count was ≤ 20 CFU/g.
- 15

Example 8

Process for producing HA, wherein *in-situ* fragmentation is carried out by cavitation (see Fig. 1)

Isolation, inoculum preparation, and main fermentation was carried out as described in **Examples 1-4**. After the fermentation broth achieved viscosity of ≥ 2000 cp after about 18 h of main fermentation, *in-situ* fragmentation was performed using ozonation (ozone gas treatment) as described in **Example 6**.

Once the viscosity of the fermentation broth is reduced to about ≤ 50 Cp, cell separation, decolorization, cation resin treatment, and solvent precipitation steps were performed as described in **Example 7** to get the final product (HA), that was analysed/characterized as described in **Example 7**.

Example 9

Screening final product (HA) for commercial applicability

The low and super low molecular weight sodium hyaluronate (HA salt) achieved by way of **Examples 7, and 8** were screened alongside cosmetic grade specifications (see **Table 13**).

[Table 13]

Sr. No.	Parameters and Reference	Test Values	Specifications
1	Appearance (Reference EP 01/2008:1472)	Almost white hygroscopic powder	A white hygroscopic powder, very hygroscopic powder, or fibrous aggregate.
2	Solubility (Reference EP 01/2008:1472)	Soluble in water, insoluble in acetone and in anhydrous ethanol	Sparingly soluble or soluble in water, practically insoluble in acetone and in anhydrous ethanol
3	Loss on drying (Reference EP 2.2.32)	15.5 %	Not more than 20 %
3	pH (Reference EP 2.2.3)	6.3	5.0 to 8.0
4	Viscosity (Reference EP 2.2.9)	0.8 m ³ /kg (<i>super low molecular weight</i>) and 1.02 m ³ /kg (<i>low molecular weight</i>)	<1.0 m ³ /kg
5	Nucleic acids (Reference EP 2.2.25)	0.019	Not more than 0.5
6	Protein (Reference EP 2.2.25)	0.1	Not more than 0.1 %

7	Assay – HA (Reference EP 2.2.25)	90.2	90 to 95 %
8	Chlorides (Reference EP 2.4.4)	< 0.5 %	Not more than 0.5 %
9	Iron (Reference EP 2.2.23)	< 80 ppm	Not more than 80 ppm
10	Molecular weight	58 kDa (<i>super low molecular weight</i>) and 710 kDa (<i>low molecular weight</i>)	<100 kDa
11	Bacterial count CFU/g	< 20	<100

As per **Table 13**, it was concluded that the low molecular weight and super low molecular weight sodium hyaluronate (HA salt) produced in the instant disclosure can be implemented for cosmetic applications.

Example 10

5 Comparative study of present process with conventional process for producing HA or salt thereof.

Comparative study between the conventional and present process is elaborated through **Table 14**.

[Table 14]

Sr. No.	Process parameter	Conventional process	Present process
1	Harvested broth quantity after fermentation	50	50
2	Ozone treatment	No	Yes
3	Water dilution	Yes	No
4	Volume of broth for centrifugation	~150	~50
5	Volume of broth for charcoal treatment	~150	~50
6	Volume of broth for cation treatment	~150	~50
7	Volume of broth for solvent precipitation	~150	~50
8	Solvent quantity	~300 (IPA)	~100 (Acetone)
9	Solvent used	IPA, EtOH, Acetone	Acetone
10	Effluent generated for wastewater treatment	~450	~150
11	Sparkler filtration system use	Yes	No
12	Molecular weight in kDa	>1500	< 800
13	Viscosity in Cp	2000 to 2500	≤ 50
14	Intrinsic Viscosity of product (m ³ /Kg)	>1.5	< 1.2
15	Culture handling sensitivity	High	Low

16	Process handling for DSP	Difficult due to higher viscosity	Easy as compared to conventional process
17	Cost of production	High due to higher quantity of chemical requirement	Low (Less chemical requirement)

As per foregoing Examples, and Comparative study, instant process demonstrates following advantages over conventional process for producing HA and/or salt thereof.

- *In-situ fragmentation (by ozonation or cavitation) achieves:*
 - 5 • *In-situ* reduction in the molecular weight of HA or salt thereof resulting in the production of stable cosmetic grade (low molecular weight and/or super low molecular weight) HA and/or salt thereof,
 - *In-situ* reduction in the viscosity of the fermentation broth resulting in the non-requirement of broth dilution step before down streaming,
 - 10 and
 - *In-situ* deactivation of majority of infectious organisms resulting in the reduction of culture handling sensitivity.
- Non-requirement of broth dilution (addition of water) step (for reducing the viscosity of the fermentation broth) before down streaming results in:
 - 15 • The reduction of process volumes,
 - The reduction of quantity of chemicals implemented during extraction and purification step (down streaming steps),
 - The reduction of effluent generated for wastewater treatment,
 - The reduction of production cost, and
 - 20 • The facilitation of feasible process handling.

The foregoing process can be tailored/regulated/modified according to the consumer and market requirements of HA or salt thereof.

The foregoing process can be applied to other HA producing *Streptococcus spp.*

25 Further, it can be carried out as continuous, semi-continuous, batch, and/or fed

batch process. It can also be implemented for small, medium, and/or large-scale production of HA or salt thereof.

5 The embodiments, examples and alternatives of the preceding paragraphs or the description and drawings, including any of their various aspects or respective individual features, may be taken independently or in any combination. Features described in connection with one embodiment are applicable to all embodiments, unless such features are incompatible.

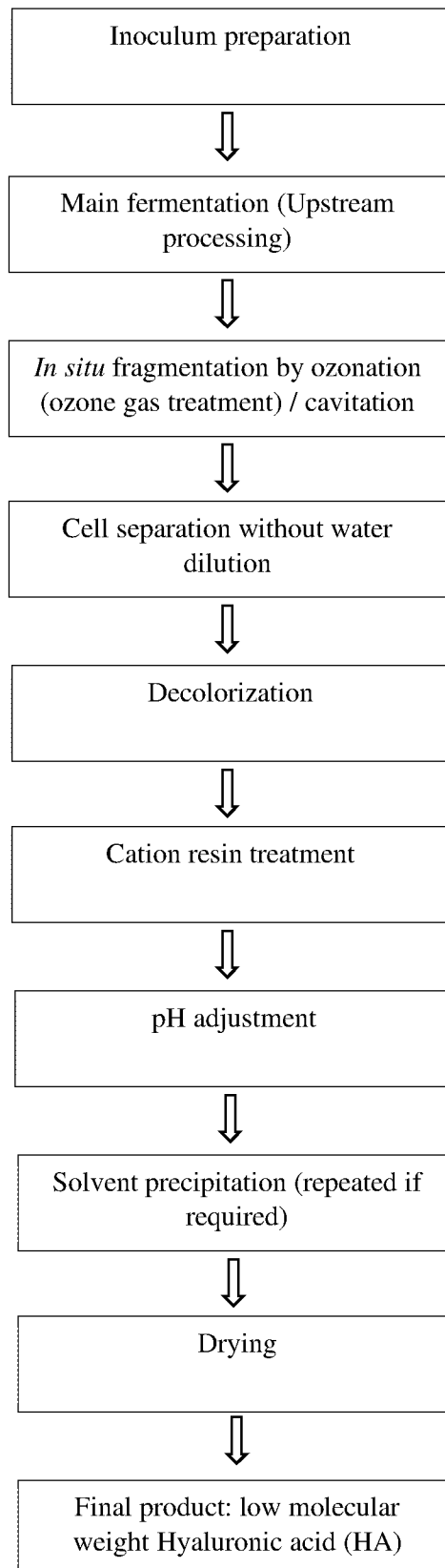
10 The preferred embodiments of the present invention are described in detail above. It should be understood that ordinary technologies in the field can make many modifications and changes according to the concept of the present invention without creative work. Therefore, all technical solutions that can be obtained by those skilled in the art through logical analysis, reasoning or limited experiments based
15 on the concept of the present invention on the basis of the prior art should fall within the protection scope determined by the claims.

WE CLAIM:

1. A process for producing low molecular weight hyaluronic acid (HA) or salt thereof, comprising:
characterized by,
5 producing high molecular weight HA or salt thereof by fermentation; and
carrying out *in-situ* fragmentation of said high molecular weight HA or salt thereof;
to obtain low molecular weight HA or salt thereof.
- 10 2. The process as claimed in claim 1, wherein molecular weight of the high molecular weight HA or salt thereof is $\geq 1800\text{kDa}$.
3. The process as claimed in claim 1, wherein molecular weight of the low molecular weight HA or salt thereof is $\leq 800\text{ kDa}$
- 15 4. The process as claimed in claim 1, wherein the fermentation is carried out in fermentation media/medium comprising at least one of dextrose monohydrate, yeast extract, sodium chloride, magnesium sulfate, dipotassium phosphate, and soya oil.
- 20 5. The process as claimed in claim 1, wherein the fermentation is carried out by *Streptococcus spp.*
6. The process as claimed in claim 1, wherein the *Streptococcus spp* are at
25 least one of *Streptococcus equi* or *Streptococcus zooepidemicus*.
7. The process as claimed in claim 1, wherein the fermentation is carried out till fermentation viscosity is $\geq 2000\text{ cp}$.
- 30 8. The process as claimed in claim 1, wherein the *in-situ* fragmentation reduces the fermentation viscosity to $\leq 50\text{ cp}$.

9. The process as claimed in claim 1, wherein the *in-situ* fragmentation is carried out by at least one of ozonation or cavitation.
10. The process as claimed in claim 9, wherein the ozonation is carried out at
5 6 lpm to 10 lpm.
11. The process as claimed in claim 9, wherein the cavitation is carried out at
2 m³/h to 52 m³/h with 2 kg/m³ to 12 kg/m³ pressure
- 10 12. The process as claimed in claim 1, wherein purity of the low molecular weight HA or salt thereof is $\geq 80\%$
13. The process as claimed in claim 1, wherein loss on drying of the low molecular weight HA or salt thereof is $\leq 20\%$
- 15 14. The process as claimed in claim 1, wherein recovery of the low molecular weight HA or salt thereof is $\geq 80\%$.
15. The process as claimed in claim 1, wherein bacterial count of the low
20 molecular weight HA or salt thereof is ≤ 20 CFU/g.
16. The process as claimed in claim 1, wherein the low molecular weight HA or salt thereof is applicable in cosmetics.

25

**Figure 1**



INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2024/052402

A. CLASSIFICATION OF SUBJECT MATTER

C08B37/08, C12P19/26, C12N1/20, C12R1/46 Version=2025.01

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)

C08B, C12P, C12N, C12R.

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

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

PatSeer, 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	IN3582/CHE/2015A (STELIS BIOPHARMA, [IN]) 20 January 2017 (20/01/2017) *claims 1-7*	1-16
Y	KR20130128655A (UNIV KOREA NAT TRANSP IND ACAD COOP FOUND [KR]) 27 November 2013 (27/11/2013) *claims 1-14*	1-16
Y	CN101294180B (JIANGNAN UNIVERSITY, [CN]) 18 April 2012 (18/04/2012) *whole document*	1-16
Y	Wu Y. Preparation of low-molecular-weight hyaluronic acid by ozone treatment. Carbohydrate polymers. 2012 Jun 20;89(2):709-12. *abstract*	1-16
Y	US11155569B2 (SHANGHAI GREEN VALLEY PHARMACEUTICALS CO LTD [CN]) 26 October 2021 (26/10/2021) *whole document*	1-16
A	Serra M, Casas A, Toubarro D, Barros AN, Teixeira JA. Microbial hyaluronic acid production: a review. Molecules. 2023 Feb 23;28(5):2084. *whole document*	1-16



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"I"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"

document member of the same patent family

Date of the actual completion of the international search

27-03-2025

Date of mailing of the international search report

27-03-2025

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2024/052402

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IN2024/052402

Citation	Pub.Date	Family	Pub.Date
US 11155569 B2	26-10-2021	AU 2017385354 A1	18-07-2019
		CA 3048734 A1	05-07-2018
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