Enhanced hyluronic acid production in *Streptococcus zooepidemicus* by over expressing HasA and molecular weight control with Niscin and glucose

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ABSTRACT

Hyaluronic acid (HA) is a high molecular weight linear polysaccharide, endowed with unique physiological and biological properties. Given its unique properties, HA have unprecedented applications in the fields of medicine and cosmetics. The ever growing demand for HA production is the driving force behind the need for finding and developing novel and amenable sources of the HA producers. Microbial fermentation of *Streptococcus zooepidemicus* deemed as one the most expeditious and pervasive methods of HA production. Herein, a wild type *Streptococcus zooepidemicus*, intrinsically expressing high levels of HA, was selected and optimized for HA production. HasA gene was amplified and introduced into the wild type *Streptococcus zooepidemicus*, under the control of Nisin promoter. The HasA over-expression increased the HA production, while the molecular weight was decreased. In order to compensate for molecular weight loss, the glucose concentration was increased to an optimum amount of 90 g/L. It is hypostatizes that excess glucose would rectify the distribution of the monomers and each HasA molecule would be provided with sufficient amount of substrates to lengthen the HA molecules. Arriving at an improved strain and optimized cultivating condition would pave the way for industrial grade HA production with high quality and quantity.

1. Introduction

Hyaluronic acid (HA, also known as hyaluronan) is a high molecular weight linear polysaccharide. HA is formed from 2000 to 25000 repeating disaccharide units of alternating D-glucuronic acid and N-acetylglucosamine moieties. The monomers of each disaccharide unit are linked by β (1–3) and β (1–4) glycosidic bonds. The HA molecule is endowed with unique physiological and biological properties including high water-holding capacity, viscoelasticity and biocompatibility. Given these properties HA has a wide range of unprecedented applications in the fields of medicine and cosmetics, like osteoarthritis treatment, ophthalmic surgery, plastic surgery, drug delivery, skin moisturizers and wound healing, to name but a few [1–3].

Extraction from rooster comb and fermentation of certain attenuated strains of group A and C Streptococcus are commonly exploited for commercial HA production. However, HA extraction from rooster combs is an arduous and costly procedure, suffering from several technical limitations, leading this method to be dwindled away [4]. On the other hands, due to existence of bacterial strains capable of superior HA productivity, fermentation process becomes more attractive for large-scale production. Non-immunogenic and non-inflammatory properties of the bacterial HA renders it as an excellent alternative medical grade HA source. Moreover, microbial HA production requires simpler downstream processing, is devoid of seasonal fluctuations, shows less batch to batch variation and reduces the risk of viral contamination. In this regard, having regulatory acceptance in both the US and UK, S. equi subsp. Zooepidemicus is among the most common strains used for fermentative HA production [5,6]. Various attempts have been addressed to increase the amount of HA, including conventional techniques (e.g. optimizing the extraction process, adapting the

culture media, and selecting strains with high HA productivity) and metabolic engineering methods [7,8].

Coming to grips with the metabolic routes involved in HA synthesis by Streptococci subsp. could play pivotal roles in the optimization of its production process. Metabolic engineering approaches could be expeditiously used to arrive at both better HA quality (controlled polymer chain length) and yield of the production process. The Streptococcus equi subsp. zooepidemicus (S. zooepidemicus) operon encodes for five genes involved in HA synthesis, namely HA synthase (hasA), UDP-glucose dehydrogenase (hasB), UDP-glucose pyrophosphorylase (hasC), a glmU paralog encoding for a dual function enzyme acetyltransferase and pyrophosphorylase activity (hasD), and a pgi paralog encoding for phosphoglucoisomerase (hasE) [9,10]. HasA is the key enzyme in the production of HA, utilizing two sugar substrates (UDP-GlcA and UDP-GlcNAc) to synthesize HA. The szHasA (hyaluronan synthase of S. zooepidemicus), which is composed of 418 amino acids, harbor's six membrane domains with the N-terminal and C-terminal ends inside the cell [11].

Although a broad wealth of Streptococcus zooepidemicus strains like S. zooepidemicus ATCC 39920 [12], Streptococcus sp. ID9102 [13], S. zooepidemicus ATCC 35426 [14], S. zooepidemicus WSH-24 [15], S zooepidemicus H23 [16], S. zooepidemicus NJUST01 [17], S. equi. ss equi CCUG 22971 (Stahl, S., US patent 2003-0134393 A1, 2003), S. zooepidemicus #104 (Shiseido, Yokohama) [18], S. zooepidemicus ATCC 35246 HA+ Lac+ Ems (1), S. equi Ferm BP-879 and S. zooepidemicus BP-878 (Morita, H., and Fuji, M., US patent 5071751, 1991), S. equi FM 100 (Hashimoto, M., Saegusa, H., Chiba, S., Kitagawa, H., Miyoshi, T., US patent 4946780, 1990) and S. zooepidemicus MTCC 3523 [19] have been subjected to HA production studies, exploring novel strains with intrinsic ability of high HA production remains highly valuable to meet the growing HA demand. In the present study, we exerted a combinatorial strategy to achieve the high quality and quantity HA. To this end, a wild type S. zooepidemicus was isolated and characterized for high level HA production, while the szHasA (hyaluronan synthase gene) was introduced into the selected strain under the control of nisA promoter and the glucose concentration was optimized for higher HA production. Ultimately, we achieved an engineered strain capable of over-expressing HasA and increased HA production yield.

2. Materials and methods

2.1. Strain and culture media

A wild type Streptococcus equi subsp. zooepidemicus (isolated and characterized from horse nasal swabs) [20] was utilized as the HA producing strain throughout this study. Stock cultures were stored at −80 °C in Tryptic soy broth medium (TBS) and 25% (v/v) glycerol, while the cultivation was in TSB at 37 °C. E.coli MC1061 strain was used as intermediate gene cloning host. Both Streptococcus equi subsp. Zooepidemicus and E.coli MC1061 strains were cultured in 2.5 μg/ml of chloramphenicol. Moreover, DH5α strain was used to contain the sequencing vector (pJET).

2.2. Molecular manipulations

Plasmid DNA isolation, agarose gel electrophoresis, restriction enzyme digestion, DNA ligation and DNA transformation were all performed by standard procedures [21] or following the specific recommendations of the manufacturer's protocol. Plasmid DNA purification kit was purchased from GeneAll biotechnology and restriction endonucleases, T4 DNA ligase, PFU polymerase and Taq polymerase were purchased from Thermo Fisher Scientific. The

electroporation was performed according to the Chen et al. [10] method with a Gene Pulser device (Eppendorf).

2.3. HasA amplification and sequencing

Genomic DNA of the wild type S. zooepidemicus was extracted using standard CTAB method and confirmed on agarose electrophoresis. Genomic sequences from Streptococcus zooepidemicus H70, CY, ATCC35246, 4047 and MGCS were analyzed for their HasA gene to find the best regions for primer design. The HasA gene was amplified from the extracted genomic DNA template, using designed primers (F-HasA: 5′- CCATGGGCAGAACATTAAAAAACCT-CATAACTG-3′ and R-HasA: 5′-TCTAGATTATAATAATTTTTTACGTG TTCCCCAGTCAGC-3′). The NcoI and XbaI restriction sites were introduced to 5′ and 3′end of the PCR product using these primers. The PCR program consisted of 1 cycle of 5 min at 95 °C, followed by 30 cycles of 94 °C (30 s), 65 °C (30 s), and 72 °C (1 min) and one final extension cycle of 72 °C (10 min). The PCR product sizes were confirmed on an agarose gel. To produce blunt-end HasA PCR product, PCR reactions were done by pfu DNA pol. Then, ligation to blunted pJET 1.2 parental vector was performed at room temperature for 10 min using T4 DNA ligase. Competent Escherichia coli DH5α cells were prepared and heat-shock transformation was performed on ampicillin selection plates. To confirm the insertion HasA containing pJET vector was purified and digested with BgLII restriction enzyme, which cuts on both sides of the insert. Gel electrophoresis (1% agarose) was performed to analyze the presence of the insert and determine its size. To identify the exact sequence of the HasA gene, it was sequenced using extracted pJET vector by MacroGen Company. The sequencing result was submitted as quarry in NCBI nucleotide BLAST tool (http://blast. ncbi.nlm.nih.gov/Blast.cgi) to determine the similarities of the HasA sequences in other S. zooepidemicus strains. The search was performed against the nucleotide collection of the BLAST tool, restricted to S. zooepidemicus strains.

2.4. Construction of recombinant strain

HasA gene was digested out of pJET plasmid with NcoI and XbaI restriction enzymes. Then, the DNA fragments were inserted into pNZ8148 expression plasmid, digested with the same restriction enzymes, by a ligation reaction to construct pNZ8148-szHas expression plasmid. The pNZ8148 and pNZ8148-szHas plasmids were then used to transform the E. coli MC1061 as an intermediate host by heat shock method. The transformants were selected on TSB plates containing chloramphenicol as selection marker. The pNZ8148 (as negative control) and pNZ8148-szHas expression plasmids subsequently were extracted and electro transformed into the S. zooepidemicus by electroporation. In order to perform the electroporation we used ice-cold cuvettes of path length of 0.1 cm, containing 50 μl of washed cells and up to 10 μl of purified plasmid DNA. Voltage was set at 1.5 kV, while the resistance was set at 200 Ω and capacitance at 25 microfarads. Immediately following 5 ms of pulse application, 1.0 ml of cold TSB broth was added to cells, which were then held on ice for 5 min prior to incubation at 37 °C for 2–3 h. The sub-cloning process was assessed by sequencing of the pNZ8148-szHas expression plasmid, extracted from the recombinant S. zooepidemicus.

2.5. HasA enzyme assay

The recombinant S. zooepidemicus strains containing pNZ8148 and pNZ8148-szHasA along with the wild type S. zooepidemicus were grown at 37 °C to an OD600 of 0.5 and induced by 20 ng/ml of Nisin. In order to express the HasA gene under the control of Nisin promoter, Nisin (Sigma, USA) was processed and used for

expression induction according to the previously described method [22]. The in vitro HasA activity was measured in disrupted cells containing the membrane-attached HasA. To perform the enzymatic reaction, cells were harvested in the exponential phase of bacterial growth and pelleted by centrifugation at 4000 rpm, 4 °C for 5 min. The supernatant was discarded, while the pellet was re-suspended in 2 ml of Phosphate- Buffered Saline (PBS, pH7.2, Invitrogen). The suspension was disrupted with ultrasonic processor on ice for 99 cycles with 60W power at 30% duty to release HasA and afterwards 0.5 ml of the obtained crude extract was added into the enzymatic reaction system (0.25 ml UDP-GlcA (4 mM in PBS, Sigma), 0.25 ml UDP-GlcNAc (4 mM in PBS, Sigma), 10 ml 1 M MgCl2 and 10 ml 0.1 M DTT). The enzymatic reaction was commenced by putting the mixed reactants in a 37 °C water bath and incubating for 2 h. The reaction was stopped by 2 min immersion into 100 °C boiling water, cooling to room temperature and subsequent addition of 1.02 ml of 0.1% SDS to free the HA molecules possibly attached on the cell debris. After 10 mins of centrifugation of the reaction solution at 4000 rpm, the supernatant was ready for the ethanol precipitation and carbazole assay. The 1 unit/mg activity of the dry cell HA synthase is equivalent to 1 mg of HA generated/min/mg of the dry cell. Therefore, one Unit/ mg dry cell activity of HasA was calculated as 1 mg HA generated per min and per mg dry cell.

2.6. HasA over expression

Proteins in the crude extract (produced as described in the previous section) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDSPAGE; 10%). Equal amounts of obtained crude extract were employed to perform standard SDS-PAGE analysis. Bradford method [23] was employed to use equal amounts of the total protein for SDS-PAGE analysis with bovine serum albumin as the standard.

2.7. Cultivation

A total of 6 batch fermentations with 2 replicates per strain were performed for wild type (WT) strain, WT strain with empty plasmid (pNZ8148) and WT strain with hasA containing plasmid (pNZ8148 + HasA) strain. Molecular weight, concentration of HA and concentration of biomass were the parameters measured and compared for all three strains. Growth experiments were conducted in a 5-l bioreactor (Model KL-5L, Iran Tajhiz Ted Co. Ltd, Iran) at a working volume of 1.2 l, and the temperature was maintained at 37 °C. The reactor was agitated at 300 rpm, and dissolved oxygen tension (DOT) studies were caried out with air or air supplemented with oxygen. The pH automatically was maintain throughout the experiment at 7 (adding NaOH and 5 M HCl).

2.8. Analytical methods

Analyzing the effects of HasA over expression on HA production, a matrix of six (10, 20, 30, 40, 50 and 60 ng/ml) different concentrations of Nisin along with two different concentrations of Glucose were used for HA production. Samples were withdrawn at regular time intervals and analyzed for Biomass amount, HA molecular weight and HA concentrations. Cell growth per hour was observed by measuring the optical density of the culture broth at 530 nm using a UV-spectrophotometer (Shimadzu, UV120-02, Japan). To measure the biomass, the optical density was measured at 530 nm and converted to biomass using the following equation [10]:

$$\text{Biomass (g/l)} = A_{530} \times 0.26 \pm 0.01$$

The viscosity of culture broth was measured using a viscometer. Molecular weight of HA was determined by measuring intrinsic viscosity [η] using capillary viscometer (Ubbelohde Dilution Capillary with 0.63-mm diameter and 5700-mm³ volume). All measurements were performed at 37 °C using 0.15 M NaCl as diluent [10]. The intrinsic viscosity [η] was assessed by British Pharmacopoeia method 2009. The average molecular weight was determined from the intrinsic viscosity using the Mark-Houwink-Sakurada equation:

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

The HA concentration was measured by carbazole method. Assessing the HA concentration, each sample from the bioreactor was first incubated with a 10% volume of 5% (w/v) SDS for 10 min to separate the cells and liberate the capsular HA. Then, the culture broth was centrifuged at 4000 rpm for 30 min. After the cells removal, 2 ml of ethanol was added to 1 ml of the supernatant from the culture broth and the solution was then refrigerated at 4 °C for 24hr to precipitate hyaluronic acid. The precipitate was recovered by centrifugation at 4000 rpm for 30 min and re-precipitated using the same procedure. Finally, the pellet was dissolved in distilled water and used for HA concentration analysis by carbazole method. To do so, disodium tetraborate solution (dissolved in sulfuric acid) was added to dissolve HA, and boiled for 15 min. After cooling to room temperature, carbazole solution was added and heated in water bath for 15 min and again cooled to room temperature. Ultimately, HA concentration was read at 530 nm with D-glucuronic acid as the standard [24]. Moreover, HA purification and its molecular weight determination was carried out using the procedure employed by Chen et al. at their respective study [10].

3. Results

3.1. HasA gene characterization

Genomic DNA of the wild type *Streptococcus zooepidemicus* was successfully isolated and observed on gel electrophoresis. The PCR reaction using designed primers resulted in a 1254-bp DNA. The amplified fragment introduced into the pJET blunt cloning vector and the cloning was confirmed by BglII restriction enzyme. The sequencing results of the HasA gene indicated a high similarity to other known HasA sequences. The BLAST search results revealed that the sequence of the wild type *Streptococcus zooepidemicus* was >97% match with the H70, CY, ATCC35246, 4047 and MGCS strains (Table 1).

3.2. HasA expression and activity assessment

The SDSPAGE results demonstrated that the HasA protein expression in the recombinant strain was enhanced (Fig. 1). The enzyme activity assay recorded up to 1.8 fold of enzyme activity augmentation for the over expressed HasA (Table 2). Moreover, Table 3 reveals that the HA production is significantly increased due to recombinant HasA activity (Table 3).

3.3. Effects of HasA over expression in different expression conditions

The evaluation results of the production concentration of biomass, HA molecular weight and the concentration of the HA are listed in Fig. 2. As the results indicated, at 40 g/L of glucose concentration increasing the Nisin concentration had positive effects on HA concentration meanwhile the molecular weight was decreased. However, at 90 g/L of glucose concentration increasing the Nisin concentration had positive effects on HA concentration while the molecular weight remained constant.

Table 1
BLAST search results. The table lists top 7 matches for the sequenced gene along with their scores. From top to down are the best matches with highest scores as compared with the strain used in this study.

| Accession	Description	Max score	Total score	Query (%)	Max identity (%)
FM204884.1	S.equi subsp. zooepidemicus H70, complete genome	2124	2124	67	97
CP001129.1	S.equi subsp. zooepidemicus MGCS10565, complete genome	2122	2122	67	97
AY173078.1	S. equi subsp. zooepidemicus hyaluronic acid operon, partial sequence	2122	2122	67	97
AF023876.1	S. equisimilis hyaluronan synthase gene, complete cds	2121	2121	67	97
CP006770.1	S.equi subsp. zooepidemicus CY, complete genome	2073	2073	67	97
CP002904.1	S. equi subsp. zooepidemicus ATCC 35246, complete genome	2073	2073	67	97
FM204883.1	S. equi subsp. equi 4047, complete genome	2073	2073	67	97

4. Discussion

The ever growing demand for HA production is the driving force behind the need for finding and developing novel and amenable sources of HA producers. Traditional methods of HA production are not tractable for industrial large scale production, therefore imminently they would be replaced by fermentative production which lacks the concerns associated with animal sourced products. Since the first reports of hyaluronic acid isolation from group A hemolytic streptococci, numerous attempts have been made to increase the amount of hyaluronan. Optimizing the extraction process, adapting the culture media, selecting strains with high hyaluronan productivity and metabolic engineering are among the most common roots of increasing the amount of hyaluronan (reviewed in [6]). In this regard, we explored a HA producing strain and bolstered the production yields both in quantity and quality, optimizing the culture condition and devising the HasA production rates.

S. zooepidemicus is among the most accepted microbial sources of HA production. Hence, selecting a locally available novel HA producing strain of *S. zooepidemicus*, capable of staggering HA synthesis, would be of great industrial interest. Given the HA yields

Fig. 1. The SDSPAGE results of HasA protein expression in the recombinant strain. Lane 1 and 2 are the recombinant strain, the lane 3 is wild type strain and the lane 4 is the protein ladder.

of previously reported studies (reviewed in [25]), the basal HA yield over 4.2 g/L prior to any optimization is surprisingly compelling for a wild type strain. This extent of metabolic adaptation for HA production provides a confounding opportunity to achieve highest yields of HA production exerting minimum optimization criteria. It has been demonstrated that HAS is responsible for glycosyl transferase activity required for polymerization of both UDP-GlcNAc and UDP-GlcUA as well as translocation of hyaluronan across the cell wall of *S. zooepidemicus* [26]. The coupled nature of HA synthesis and translocation allows the HA molecule to be synthesized even in the presence of a large excess of HA-degrading enzyme. Moreover, HasA have a determinant role in the mechanism and amount of HA elongation [27]. Considering the imperative roles of HasA in the HA production process, extrapolating better HA yields would be conceivable by increased levels of HasA activity. Chen et al. cloned and over expressed five genes of the HasA operon in *S. zooepidemicus*, to manipulate the levels of UDP-GlcUA and UDP-GlcNAc. They demonstrated that over expression of the HA synthase gene (HasA) results in a significant increase in HA yield. They also reported that HasA over expression was associated with 47% increase in yield, a significant lowering of molecular weight and decrease of microbial growth rate. Thus, establishing a recombinant strain with controlled HasA over expression possibility would seems to be rational. In line with this hypothesis, our results indicate that Nisin mediated induction of HasA resulted in higher HA yield. However, the higher concentrations of Nisin induction reduce the amount of HA production. This could be a consequence of Nisin toxicity for cells and its inhibitory effect on bacterial growth [28]. The HasA gene is subcloned under the control of the Nisin promoter of the pNZ8148 plasmid, therefore HasA is the only overexpressed gene. This gene is responsible for the linkage between N-acetyl glucosamine and glucuronic acid for HA production which does not play any other role.

Pummill and Deangelis testified the theory that the relative strength of the interaction between the catalyst and the precursor sugars may be a major factor in HA size control via in vitro confirmation [29]. Later on, Sheng et al. discovered the applicability of this theory in their in vivo experiments [30]. They hypothesized that altered distribution of precursor sugars among the HasA protein molecules could be the reason behind this phenomena. They suggested that, HasA is essential, but not sufficient for high field HA production in heterologous hosts and the production of UDP-GlcA is the limiting factor in host cell. As an alternative strategy to enhance UDP-GlcNAc concentration, Chen et al. suggested feeding the culture with glucosamine. As it was expected, HasA over expression decreased the HA molecular weight in our experiment. Our results indicate that HasA overexpression in 40 g/l of glucose concentration is associated with increased amount of HA, while MW is decreased. This could be the consequence of altered distribution of precursor sugars, so that each HA polysaccharide chain gets less precursor sugars to lengthen itself by HasA. However, to compensate the MW reduction glucose concentration was contemplated to be
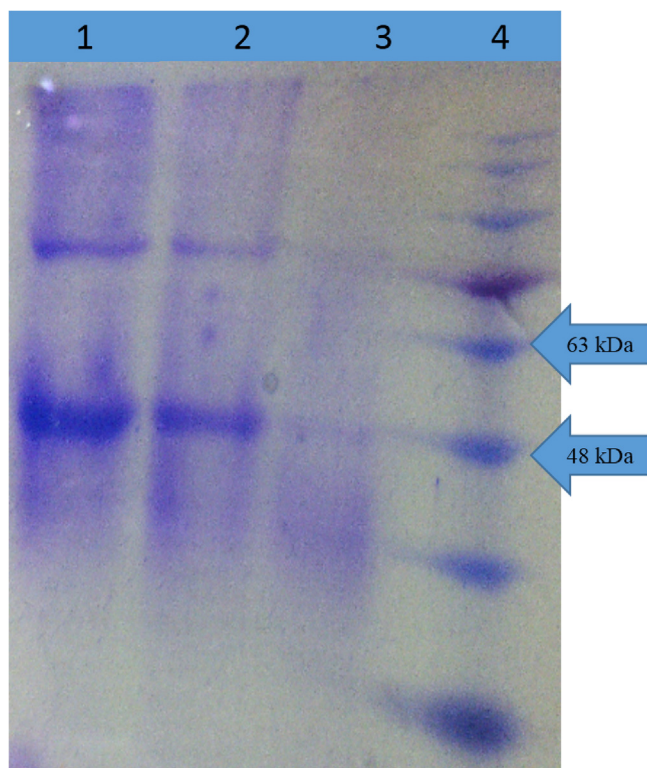
Table 2
Activity of HasA enzyme in comparison to wild type strain ($p \leq 0.05$).

Fold increase in comparison to WT	Specific activity mg HA min^{-1} mg protein^{-1}	Strain
1	$0.49 \times 10^{-3} \pm 0.01$	WT(S.zooepidemicus)
1.02	$0.50 \times 10^{-3} \pm 0.01$	RM(pNZ8148)
1.82	$0.89 \times 10^{-3} \pm 0.02$	RA(pNZ8148 + HasA)

Table 3
The variation in HA concentration comparing wild type and recombinant strains ($p \leq 0.05$).

Percentage of HA increase in comparison to WT	HA concentration (g/l)	Strain
0	4.20 ± 0.01	WT(S.zooepidemicus)
0	4.24 ± 0.01	RM (pNZ8148)
43	6.02 ± 0.02	RA(pNZ8148 + HasA)
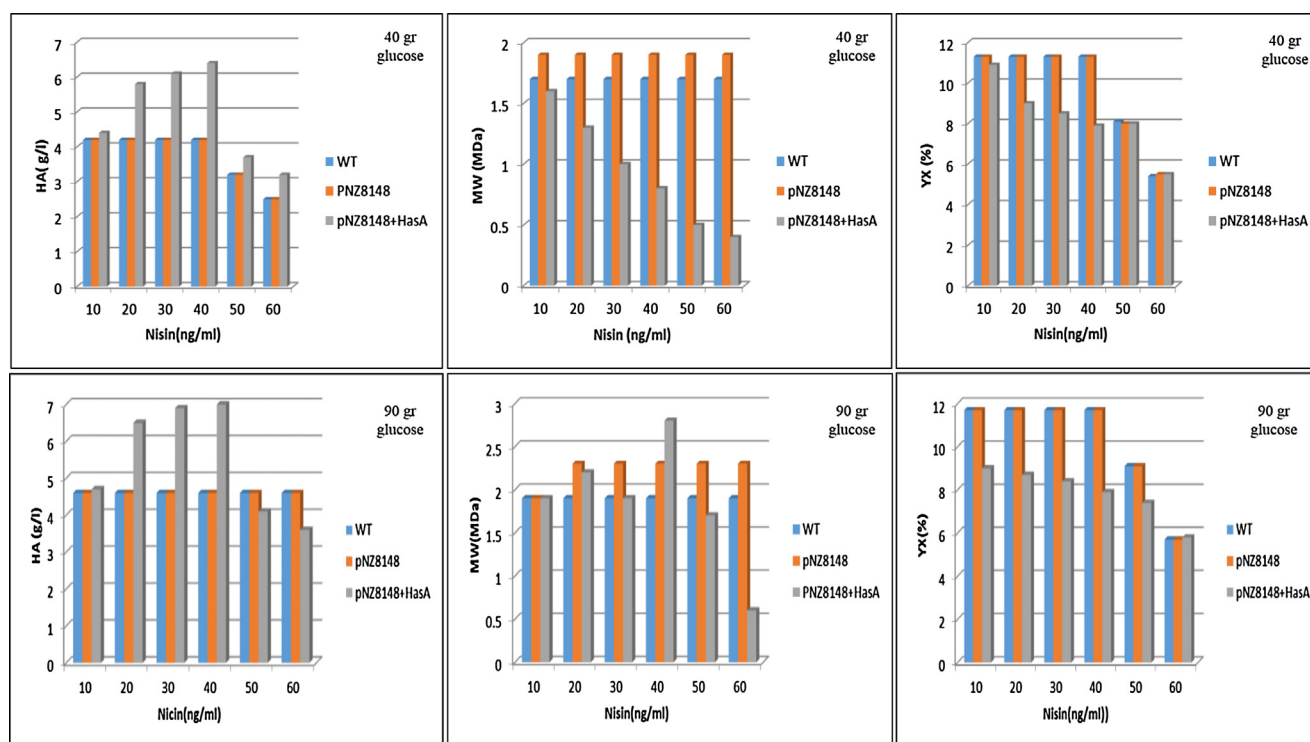
Fig. 2. The HA fermentation values for different Nisin and glucose concentrations. WT stands for wild type, pNZ8148 stands for WT strain with empty plasmid, pNZ8148 + HasA stands for WT strain with hasA containing plasmid strain, MW is molecular weight and YX is yield of biomass.

optimized. Additional Glucose would provide the host with sufficient amount of UDP-GlcA, as the limiting factor. Therefore, suitable distribution of sugar monomers would occur among the HasA proteins and MW would increase. Our results revealed that HasA over-expression along with increased glucose resources up to 90 g/L would lead to increased HA production, while the MW is relatively kept constant in comparison to wild type strain. It should be noted that, the molecular weight of the HA at 40 gr/L glucose concentration of the wild type bacteria is 1.7 million Da, while at the 90 gr/L glucose concentration the molecular weight of the HA molecule reached 1.9 million Da. Therefore, it could not be concluded that the molecular weight of HA is constant during glucose fluctuation. On the other hands, Nisin increase does not have a tangible effect on molecular weight. Since, aside from the HA production, glucose is involved in energy production processes, only 5% of the glucose is consumed in HA production. Therefore, it is anticipated that it does not play a pivotal role in molecular

weight variation and Nisin plays a more fundamental role. The study conducted by Chen et al. revealed that the increase in MW could be partially due to the foreign DNA stress exerted by existence of the plasmid within the bacteria. They demonstrated that the plasmid backbone without any external genes could surprisingly increase the MW. In fact, there are evidences indicating stress in general (plasmid stress, aerobic conditions, changes in pH, and low temperature) is beneficial for high molecular weight HA production [10]. It is noteworthy to mention that, the fermentation process is performed by an automatic fermenter which its oxygen sensor was active during the whole process. The oxygen concentration was automatically fine-tuned throughout the process. Due to exerted control, the oxygen concentration was kept constant; thus the possible influences of dissolved oxygen concentration have been eliminated in the experiments. In conclusion, it should be noted that optimization of culture media and cultivation conditions along with strain

improvements are intriguing methods, harnessed for improving HA production yield. Performing these improvement on a wild type strain which is already capable of high yield HA production would circumvent the common obstacles lies ahead of industrial level fermentative HA production. Having an improved strain and optimized culture media would pave the way for industrial grade HA production with high quality and quantity.

Conflict of interest

The authors declare no conflict of interests.

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