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ORIGINAL PAPER

Evaluation of Sheep Wool Protein Hydrolysate and Molasses as Low-Cost Fermentation Substrates for Hyaluronic Acid Production by *Streptococcus zooepidemicus* ATCC 35246

Nazli Pinar Arslan[1] · Mehmet Nuri Aydogan[2]

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Abstract
Peptones are widely used as complex fermentation substrate for hyaluronic acid (HA) production; however, use of peptones in HA production reduces commercial competitiveness due to their high price. The present study was conducted to test the feasibility of sheep wool peptone (SWP) (mainly organic nitrogen source) and molasses (mainly carbon source) as cheap substrates for HA production from *Streptococcus zooepidemicus* ATCC 35246. Six peptones (SWP I–VI) were prepared from sheep wool using different chemical hydrolysis methods. Among them, SWP-VI was determined to be more fovarable for HA production. SWP-VI was compared with commercial tryptone peptone (TP) and protease peptone (PP) in order to evaluate its effectiveness in production of HA, lactic acid (LA) and cell biomass (CB). The protein contents of SWP-VI, TP and PP were determined as 70.6, 83.1 and 83.8 g/100 g, respectively. The best peptone for HA and CB production was SWP-VI, whereas PP was found to be more favourable for LA production. Maximum HA concentrations in SWP-VI, TP and PP media were determined as 3.54, 2.58 and 2.47 g/L, respectively.

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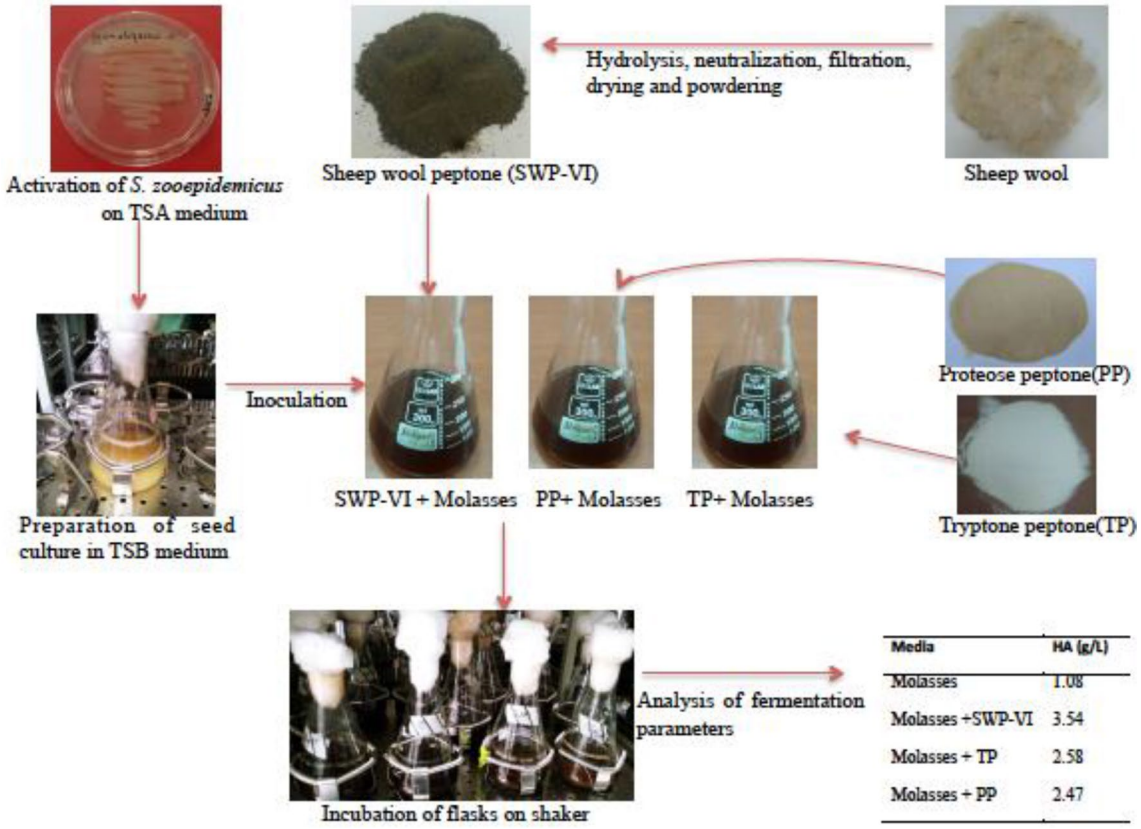
✉ Nazli Pinar Arslan
nparslan55@hotmail.com

[1] Vocational School of Health Services, Bingol University, Bingol, Turkey

[2] Department of Biology, Science Faculty, Ataturk University, Erzurum, Turkey

Graphic Abstract



Keywords *Streptococcus zooepidemicus* · Sheep wool peptone · Molasses · Hyaluronic acid

Statement of Novelty

This is the first attempt on the evaluation of keratinous materials including sheep wool as organic nitrogen or peptone source for fermentative production of HA. The prepared sheep wool peptone can be also employed as substrate in future studies for the production of other industrially and/or biotechnologically important substances such as polysaccharides, organic acids, probiotics, recombinant proteins and single cell protein.

Introduction

Hyaluronic acid (HA) is a high molecular mass polymer formed by repeating disaccharide units of Nacetyl-D-glucosamine and D-glucuronic linked by β(1–3) and β(1–4) glycosidic bonds [1]. HA is found in vertebrates (such as human and rooster) and in the capsules of some bacteria (e.g., strains of *Streptococci*), but is absent in fungi, plants, and insects [2]. In the human body, HA is widely present in the eyes, skin, cartilage, heart, inter-vertebral discs of the spine and in the fluids of the middle and inner ear [2, 3].

This polymer has some important physicochemical and biological properties in human body, such as lubricity, viscoelasticity, water holding capacity and biocompatibility [1]. Due to these potential properties, exogenous HA is extensively used in cosmetic and biomedical industries [2–5].

The first HA used for clinical purposes was extracted from the umbilical cords and rooster combs [6]. However, there are some concerns related with the use of rooster combs-based HA for biomedical and pharmaceutical applications [7]. For example, rooster combs-based HA products used for human therapeutics cause viral agent infections. On the other hand, the purification of HA from rooster combs is a difficult process [8]. Therefore, microbial fermentation has emerged as a new alternative approach for HA production.

The most preferred strain in fermentative production of HA is *Streptococcus zooepidemicus* [7]. However, it has been reported that the growth and HA production potential of *S. zooepidemicus* is highly depended on organic nitrogen

as well as some vitamins and minerals. Especially with respect to organic nitrogen, the bacterium has fastidious nutrient requirements [1, 9–11]. Therefore, yeast extract and/or peptone are extensively added as organic nitrogen into the culture medium of *S. zooepidemicus*. For example, it is stated that this bacterium needs very high concentrations (up to 20 g/L) of peptone and/or yeast extract for HA production [12–14]. However, it is well known that high price of peptones and/or yeast extract decrease the commercial competitiveness of microbial fermentation in HA production. To solve the cost problem, some investigators have suggested using low-cost renewable resources and agro-industrial by-products as peptone or organic nitrogen source. For instance, Amado et al. [11, 15] demonstrated that cheese whey and corn steep liquor might be alternative organic nitrogen source for HA production from *S. zooepidemicus*. In other two studies [1], it was demonstrated that peptones or protein hydrolysates from fish wastes could be employed as cheap organic nitrogen sources for HA production from *S. zooepidemicus*. Apart from these two studies, there are no other studies in the literature on the development of alternative peptone source for HA production. Therefore, there is a need for new studies for the discovery of low cost peptones that may be an alternative to commercial peptones in fermentative HA production.

Keratin proteins are the major components of hair, feathers, wool and horns [16]. Sheep wool has an approximately protein content of 97% (82% keratin protein) and contains 20 different amino acids [17–19]. Recenty, it has been documented that protein hydrolyzates prepared from sheep wool by chemical and/or enzymatic methods can be used as plant fertilizer and animal feed [17, 20–23]. Taskin and coworkers [24] have also demonstrated that sheep wool protein hydrolyzate (peptone) can be also employed as a general growth substrate for some microorganisms (*Bacillus subtilis, Esherichia coli, Staphylococcus aureus, Aspergillus niger, Penicillium chrysogenum* and *Saccharomyces cerevisia*). However, their study was focused on investigating the only growth performance of these microorganisms on sheep wool peptone (SWP). To our best knowledge, there is no report on the use of SWP as substrate or organic nitrogen source for the fermentative production of valuable substances such as organic acids, polysaccharides, single cell protein, pigments, ethanol, enzymes, vitamins and antibiotics. In this regard, it is possible to say that SWP may a good alternative nitrogen source for HA production in the growth medium of *S. zooepidemicus*.

Molasses is a by-product that remains after the extraction of sugar from sugar beet or sugar cane. Sugar beet molasses contain 23–26% water, 47–48% sugar, 9–14% minerals and 8–12% nitrogenous compounds (aminoacids, proteins, etc.) [25, 26]. Due to rich nutritional composition, it is widely used as a fermentation substrate for production of industrially and/or biotechnologically valuable substances such as organic acid, single cell protein and biohydrogen [27–29]. Furthermore, molasses is reported to be used as substrate (mainly carbon source) in HA production by *S. zooepidemicus* [11, 30, 31]. However, molasses and sheep wool were not used together as substrate in HA production. Their usage as substrate in microbial media reduces the cost of production process. Moreover, use of molasses in microbial fermantations can make a significant contribution to the reduction of the environmental pollution problem. Because, it is well known that molasses can cause some detrimental effects on the ecosystem. For instance, when molasses is discharged to soil, it prevents seed germination and reduces soil alkalinity. It limits photosynthesis by preventing sunlight from entering the lower layers in aquatic systems [32, 33].

Considering the drawbacks mentioned above, the present work was performed to (1) prepare peptone from sheep wool and (2) produce HA from *S. zooepidemicus* using wool peptone and molasses as cheap fermentation substrates.

Materials and Methods

Preparation of Different Wool Peptones

Preparation of peptones from sheep wool was performed with some modifications according to previous studies [24, 34]. Firstly, sheep wool was washed with deionized water and then dried to constant weight in an oven at 100 °C. Dried wool (100 g) was hydrolyzed in 150 mL of acidic or alkaline solution inside a heat-resistant bottle. When wool hydrolyis was perfomed using 6 N solution of HCl, H_2SO_4 or H_3PO_4, the neutralization of the prepared hydrolysate was performed using the alkaline solution which contained 10 N KOH and 10 N $Mg(OH)_2$. When wool was hydrolyzed using 2.5 N KOH, NaOH or $Mg(OH)_2$, the prepared hydrolysate was neutralized using 6 N H_3PO_4.

After the acidic or alkaline hydrolysates were prepared as described above, they were subjected to neutralization, filtration, evaporation and drying processes, respectively. In this way, the production of different sheep wool peptones (SWPs) was aimed. The production scheme of SWPs was summarized in Supplementary Material Fig. S1.

Determination of Chemical Composition of Molasses, Different Wool Peptones and Commercial Peptones

Total nitrogen content of molasses, SWPs and commercial peptones was determined using a micro-Kjeldahl apparatus (Labconco Corporation, Kansas City, MO, USA), and crude protein content was estimated by multiplying the nitrogen contents by 6.25. Ash contents were determined by

combusting a dry sample for 3 h in a muffle furnace (Thermolyne 62700, Barnstead/Thermolyne Corp., Dubuque, IA, USA) at 550 °C. Their elemental compositions were determined by using an Inductively Couple Plasma spectrophotometer (ICP-MS) (Agilent 7800). Total sugar contents were determined according to phenol–sulphuric acid method [35]. Amino-acids analyses of commercial peptones and the best SWP were carried out using LC–MS (Agilent 6460 Triple Quadrapole). Their total lipid contents were determined according to the Soxhlet extraction method using diethyl ether as solvent.

Preparation of Pre-culture

The strain *Streptococcus equi* subsp. *zooepidemicus* ATCC 35246 was selected for the present experiments. The preculture of the bacterium was prepared in a 250-mL flask containing 100 mL of standard trypticase soy broth (TSB) medium. To do this, the medium inside flask was sterilized and then inoculated with a loopfol of cell biomass of the bacterium growing on trypticase soy agar (TSA) medium. Afterwards, the inoculated flask was incubated at 200 rpm on a shaking incubator at 37 °C. After an incubation period of 24 h, 1 mL of the prepared pre-culture (about 2.0 absorbance at 600 nm) was employed for the inoculation of hyaluronic acid (HA) production medium.

Hyaluronic Acid Production in Shaking Flask Culture

Preliminary experiments for HA production were focused on determining the optimal molasses concentration. In this stage, the experiments were carried out in the liquid medium containing only molasses (at the different concentration from 6% to 20) and no other additives such as nitrogen and minerals.

After the optimal molasses concentration was determined, the following experiments were performed to determine the best SWP for HA production. In this stage, the production medium contained molasses (at the optimal concentration) and any of different SWPs (at the concentration of 4 g/L). After the most favorable SWP for HA production was determined, it was compared with two commercial peptones (TP, tryptone peptone and PP, protease peptone) at the different concentrations from 4 to 20 g/L.

All the experiments for HA prdoduction were performed inside 250-mL flasks containing 100 of the production medium (pH 8.0). For this purpose, the flaks were covered with cotton plugs, sterilized in autloclave and cooloed at the room temperature. After inoculating with one mL of preculture, the flasks were incubated at 200 rpm on a shaking incubator at 37 °C.

Analysis of Fermentation Parameters

A sample (10 mL) taken from culture broth was mixed with 10 mL of 0.1% SDS. The mixture was incubated at room temperature for 10 min to remove capsular HA from cells. Afterwards, the mixture was centrifuged at 6000 rpm for 10 min to precipitate cells. The obtained pellets were washed and then dried at the constant weight at 100 °C in order to determine cell biomass (CB). The supernatant was employed for estimating HA, lactic acid (LA), residual sugar (RS) and pH. LA was determined using HPLC (Shimadzu Prominence LC-20A). Residual sugar was determined according to phenol–sulphuric acid method [35] using sucrose as standard.

For the partial purification of HA, 5 mL of supernatant was firstly mixed with 15 mL ethanol and the mixture was then incubated for 24 h at 4 °C. At the end of incubation period, the precipitate was collected by centrifugation (3000 rpm for 20 min at 4 °C). Then, 25 mL of ethanol-saline water solution (75% ethanol, 25% 0.15 M NaCl, w/v) was added onto the precipitate in the centrifuge tube. Afterwards, the tube was re-centrifuged (3000 rpm, 4 °C, 20 min) and the final precipitate was dissolved into 30 mL of 0.15 M NaCl solution [36, 37]. This solution was used for estimating HA concentration. HA assay was performed with some modifications according to previous reports [38, 39]. For this, 1 mL of 0.15 M NaCl-HA solution was introduced into glass tubes filled with 5 mL of 25 mM Na_2B4O_7 in H_2SO_4. Each tube containing this reaction mixture was incubated for 15 min at 95 °C and then cooled in ice for 2 min. Afterwards, 0.2 mL of 0.125% (w/v) carbazole in absolute ethanol were added into this mixture, and each tube was re-incubated for 15 min at 95 °C and then cooled in ice for 2 min. The absorbance of the mixture was read at 550 nm using a spectrophotometer. Standard graphic was prepared using commercial HA. During the assay, the mixture containing 1 mL of 0.15 M NaCl solution was used as the blank.

Statistical Analysis

The elemental and amino-acids analysis of peptones were done in two technical replicates, and average values were given. The other analyzes (HA, CB, RS, LA, RS and pH) were performed in triplicate and the results were given as the mean of the three values ± standard deviation (SD). Whether the obtained data is statistically significant or not has been determined in the SPSS 15.0 package program using $P < 0.05$ significance level one-way analysis of variance (ANOVA) and Duncan's Multiple Comparison Test.

Results and Discussion

Preparation of Sheep Wool Peptone

Peptones or protein hydrolysates are prepared from organic materials using chemicals and/or enzymes. Especially strong acids (H_2SO_4 and HCl) or alkalines (NaOH and KOH) are mostly preferred for hydrolysis of keratinous materials such as feather and wool. The prepared hydrolysates are then neutralized using acids or bases [17, 24, 34, 40]. Therefore, three strong acids (H_2SO_4, H_3PO_4 and HCl) or three bases (NaOH, KOH and $Mg(OH)_2$) were tested for hydrolysis of sheep wool in this work. The obtained hydrolysates were neutralized using acidic or alkaline solution. In this way, production of six different sheep wool peptones (SWPs) was achieved. They were listed below.

Sheep wool peptone I (SWP-I) 6 N H_2SO_4 was used for hydrolysis, the obtained hydrolysate was then neutralized using the alkaline mixture containing 10 KOH and 10 N $Mg(OH)_2$.
Sheep wool peptone II (SWP-II) 6 N HCl was used for hydrolysis, the obtained hydrolysate was then neutralized using the alkaline mixture containing 10 KOH and 10 N $Mg(OH)_2$.
Sheep wool peptone III (SWP-III) 6 N H_3PO_4 was used for hydrolysis, the obtained hydrolysate was then neutralized using the alkaline mixture containing 10 KOH and 10 N $Mg(OH)_2$.
Sheep wool peptone IV (SWP-IV) 2.5 N $Mg(OH)_2$ was used for hydrolysis, the obtained hydrolysate was then neutralized using 6 N H_3PO4.
Sheep wool peptone V (SWP-V) 2.5 N NaOH was used for hydrolysis, the obtained hydrolysate was then neutralized using 6 N H_3PO_4.
Sheep wool peptone VI (SWP-VI) 2.5 N KOH was used for hydrolysis, the obtained hydrolysate was then neutralized using 6 N H_3PO_4.

The previous studies demonstrated that three elements (K, P and Mg) significantly augment HA synthesis in *S. zooepidemicus* strains [9, 14, 41]. Hence, in the present study, the acids and/or bases incorporating these elements into the peptones were selected for hydrolysis and/or neutralization steps. For instance, the neutralization of the hydrolysate prepared using H_2SO_4 was performed using KOH and $Mg(OH)_2$ in order to increase K and Mg contents (SWP-I). Similarly, it was aimed that SWP-III became richer in terms of K, Mg and P by selecting suitable hydrolyzing or neutralizing agents such as H_3PO_4, KOH and $Mg(OH)_2$. Taking into account of the chemicals selected for hydrolysis and neutralization steps, it was considered that SWP-III would be more suitable for HA production since it contained all of K, P and Mg elements. But, the experiments demonstrated that only 10 g of 25 g wool could be hydrolyzed using H_3PO_4. Namely, the hydrolysis yield obtained with H_3PO_4 was too low. Similarly, too low hydrolysis yield (only 5.3 g of 25 g wool) was achieved when wool was subjected to $Mg(OH)_2$ hydrolysis (SWP-IV). Therefore, it was considered that the processes designed for production of SWP-III and SWP-IV were not suitable. Conversely, favorable hydrolysis yields were obtained using HCl, H_2SO_4, NaOH or KOH. About 82.2 g of 100 g wool was hydrolyzed using H_2SO_4 (SWP-I). When HCl was used, 94.2 g of 100 g wool was hydrolyzed (SWP-II). NaOH could hydrolyze about 90 g of 100 g wool (SWP-V). When KOH was selected, 93.2 g of 100 g wool could be hydrolyzed (SWP-VI).

Based on these results, the hydrolysates which were prepared with HCl, H_2SO_4, NaOH or KOH hydrolysis were considered to be suitable for preparing peptone. Therefore, these hydrolysates were then subjected to neutralization, filtration and drying processes, respectively. At the end of these steps, 129.4 g SWP-I, 142.4 g SWP-II, 116.1 g SWP-IV and 118.2 g SWP-VI could be prepared (Supplementary Material Table S1). These results demonstrated that the most produced peptone was SWP-II among four peptones. This situation could be ascribed to the use of much more acids and bases for production of SWP-II. This assumption was also supported when the chemical analysis of peptones were performed. Namely, the highest ash content (37.1 g/100 g) was determined for SWP-II. The protein contents of peptones increased as the ash contents decreased. Therefore, the highest protein content (70.6 g/100 g) was recorded for SWP-VI having the lowest ash content (24.1 g/100 g). The protein contents of SWP-I, SWP-II and SWP-IV were determined as 65.4, 57.4 and 67.5 g/100 g, respectively (Supplementary Material Tables S1 and S2).

Hyaluronic Acid Production in Molasses Medium

Preliminary studies on HA production were performed in the medium containing only molasses. The total sugar, protein and ash contents of molasses were determined as 47.4, 10.0 and 8.2 g/L, respectively.

When the molasess alone was used in the medium, the maximum production of HA (1.08 g/L) and cell biomass (CB) (2.87 g/L) could be achieved at 14% molasses concentration. At this molasses concentration, the sugar content of the production medium was calculated as 66.3 g/L. Higher concentrations of molasses gave rise to the reductions in HA and CB concentrations (Fig. 1). This inhibitory effect could be attributed to the presence of some phenolics compouns and excessive metal ions inside molasses.

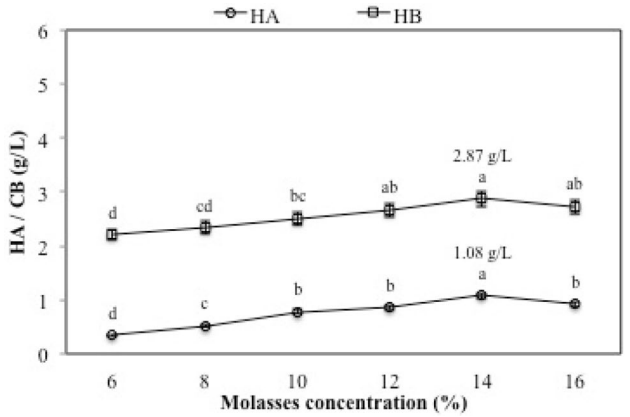


Fig. 1 Effect of different molasses concentrations on HA and CB production in *S. zooepidemicus* ATCC 35246. The experiments were performed in the medium (control medium) containing only molasses. Initial pH 8.0 and incubation time 48 h. HA, hyaluronic acid and CB, cell biomass. All the measurements were taken in three replicates. Error bars represent standard deviation of replicates. Different lowercase letters in the line of HA or CB production indicates significant differences ($P < 0.05$)

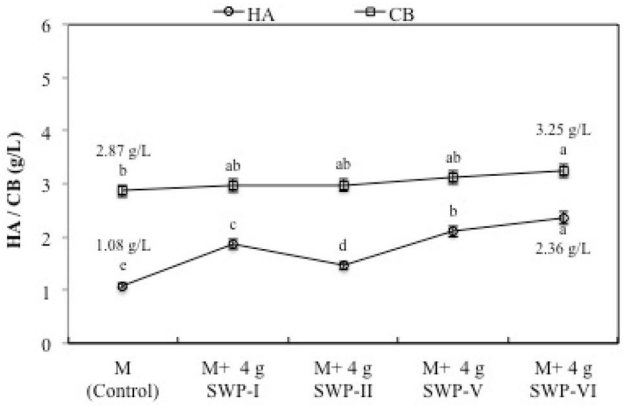


Fig. 2 Effect of different SWPs on on HA and CB production in *S. zooepidemicus* ATCC 35246. The experiments were performed in the media containing 14% molasses and 4 g/L SWP. Initial pH 8.0 and incubation time 48 h. *SWP* sheep wool peptone, *HA* hyaluronic acid and *CB* cell biomass. All the measurements were taken in three replicates. Error bars represent standard deviation of replicates. Different lowercase letters in the line of HA or CB production indicates significant differences ($P < 0.05$)

This is because some phenolic compounds inside molasses have been reported to show antibacterial effect against some *Streptococci* species [42]. In another study [31], it was demonstrated that when molasses was detoxified using activated carbon, about %19 increase in HA and CB production was achieved. On the other hand, it is well known that sucrose accounts for about 90% of total sugar content of molasses. Considering this knowledge, total sugar and sucrose contents of 16% molasses was calculated as 75.7 and 68.2 g/L, respectively. The previous studies have shown that sucrose concentrations above 60 g/L limit the growth and HA production potential of *S. zooepidemicus* [11, 30, 43, 44]. So it was concluded that high sucrose content in molasses of 16% was another reason of the inhibitory effect on HA and CB. Based on these results, the subsequent experiments were performed at the optimal molasses concentration of 14%.

Selection of the Best Sheep Wool Peptone for HA Production

In the second stage of HA production, the molasses medium was supplemented with any of four different SWPs (SWP-I, SWP-II, SWP-IV and SWP-VI) at the same concentration (4 g/L). The experiments revealed that the peptones (SWP-I and SWP-II) prepared with acid hydrolysis showed poor solubility in the medium (initial pH of media was pH 8.0). Namely, a large amount of peptone-derived solid precipitate occurred in the medium and the precipitate became insoluble during the incubation period. Conversely, the peptones (SWP-IV and SWP-VI) prepared using alkaline hydrolysis were found to be completely dissolved in the medium (Supplementary Material Table S1).

As seen from Fig. 2, the addition of SWPs to the medium exerted more positive effect on HA synthesis rather than cell growth. The maximum HA production (2.36 g/L) was achieved in the medium supplemented with SWP-VI. The second highest HA concentration (2.11 g/L) could be reached in SWP-IV medium. These results were probably due to higher solubility and/or richer protein contents of SWP-IV and SWP-VI. The other possible reason was high P content of SWP-IV and SWP-VI, since H_3PO_4 (P source) was used as neutralizing agent for their preparation. Taking into account of these results, SWP-VI was selected as the best sheep wool peptone for the following experiments.

Comparison of Chemical Compositions of SWP-VI and Commercial Peptones

Chemical analyses demosnstrated that protein content (70.6 g/100 g) of SWP-VI was lower than TP (83.1 g/100 g) and PP (83.3 g/100 g). Contrary, the highest ash content (24.1 g/100 g) was determined for SWP-VI (Supplementary Material Table S2). Low protein and high ash contents of SWP-VI could be ascribed to the excessive use of chemicals during preparation of SWP-VI. Namely, acids and bases which were used in hydrolysis and neutralization steps lowered the protein content by increasing ash content, as noted in the previous studies [34, 45].

The elemantal analysis demosntrated that Na content of SWP-VI was lower than those of other peptones, whereas K, P, Mg, Fe and Ca contents of SWP-VI were higher than

those of TP and PP (Supplementary Material Table S3). K and P contents of SWP-VI were 14,224 and 6014 mg/100 g, respectively. High P and K contents in SWP-VI were mainly associated with use of H_3PO_4 and KOH. Although $Mg(OH)_2$ was not used in the preparation of SWP-VI, a high amount of Mg in SWP-VI was determined. This result was ascribed to the chemical composition of sheep wool. On the other hand, amino-acids analyzes demonstrated that when compared to the commercial peptones, SWP-VI had higher contents of six amino-acids (arginine, tyrosine, threonine, proline, serine and cystine) (Supplementary Material Table S4).

Determination of the Optimum Concentration of SWP-VI and Commercial Peptones for HA Production

In this stage of the study, the effectivenes of SWP-VI on HA production was compared with that of two commercial peptones (Tryptone peptone, TP and Protease peptone, PP). For this purpose, optimal concentrations of SWP-VI, TP and PP were firstly investigated. As can be seen from the results presented in Fig. 3, the optimal concentration of SWP-VI for HA production (3.54 g/L) was 12 g/L, whereas the optimal concentration of both TP and PP for HA production (respectively 2.58 and 2.47 g/L) was determined as 8 g/L. This difference in optimal concentration was probably due to the protein contents of peptones. Namely, because protein content of SWP-VI is lower, it was required to add more SWP-VI to the medium.

On the other hand, peptones concentrations above optimal sharply decreased HA production. These results could be ascribed to the the carbon/nitrogen (C/N) ratio of the medium, since it is widely reported that C/N ratio significantl affect HA synthesis in *S. zoopedimicus* strains [46, 47]. As in HA synthesis, cell growth was also positively affected by peptone addition. However, this positive effect on cell growth was more restricted. For example, addition of 12 g/L SWP-VI caused to about 227% increase in HA production but only 45% increase in CB production, when compared to the control medium.

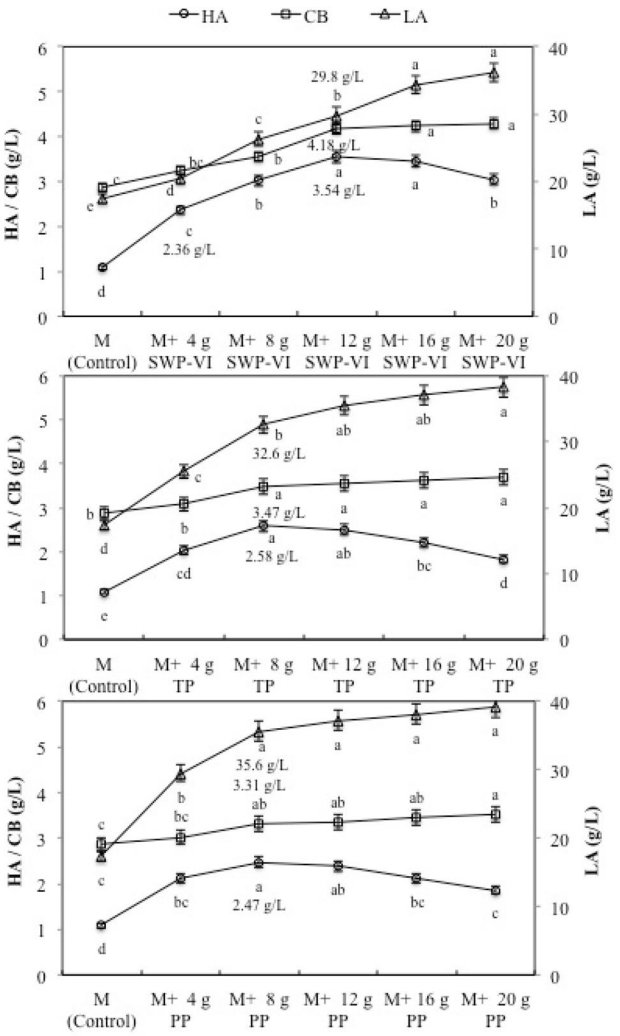


Fig. 3 Effect of different concentrations of SWP-VI, TP and PP on HA, LA and CB production in *S. zooepidemicus* ATCC 35246. The experiments were performed in the media containing 14% molasses and (4–20 g/L) peptone. Initial pH 8.0 and incubation time 48 h. *SWP* sheep wool peptone, *TP* tryptone peptone, *PP* protease peptone, *LA* lactic acid, *HA* hyaluronic acid and *CB* cell biomass. All the measurements were taken in three replicates. Error bars represent standard deviation of replicates. Different lowercase letters in the line of HA, LA or CB production indicates significant differences ($P < 0.05$)

Time Profile of Sugar Consumption, pH Change, Cel Growth, Hyaluronic Acid Synthesis and Lactic Acid Synthesis in Different Peptones Media

As seen from Fig. 4, the highest increases in the production of CB, HA and LA in the control medium (only %14 molasses) occurred between 24–36 h and their maximum concentrations were reached after 60 h. At the end of 60-h optimal incubation period, the maximum concentrations of HA, CB and LA were determined as 1.14, 3.14 and 14.9 g/L, respectively. Similarly, although sugar consumption and pH drop in the control medium continued until 60th h, both of them were faster between 24–36 h. At the end of 60th h, the concentration of residual sugar (RS) was 14.7 g/L and the final pH was 5.8. After 60th h, CB concentration decreased but HA, LA and RS concentrations as well as culture pH did not change. The decrease in CB concentration could be attributed the death of bacterial cells due to the deficiency of nitrogen source in the molasses.

In SWP-VI (Fig. 5), TP (Fig. 6) and PP (Fig. 7) media, the highest increases in HA, HB and LA concentration and as well as sugar consumption was detected between 12–24 h and their concentrations reached the maximum at the end of 48th h. In the peptone media, sugar consumption was

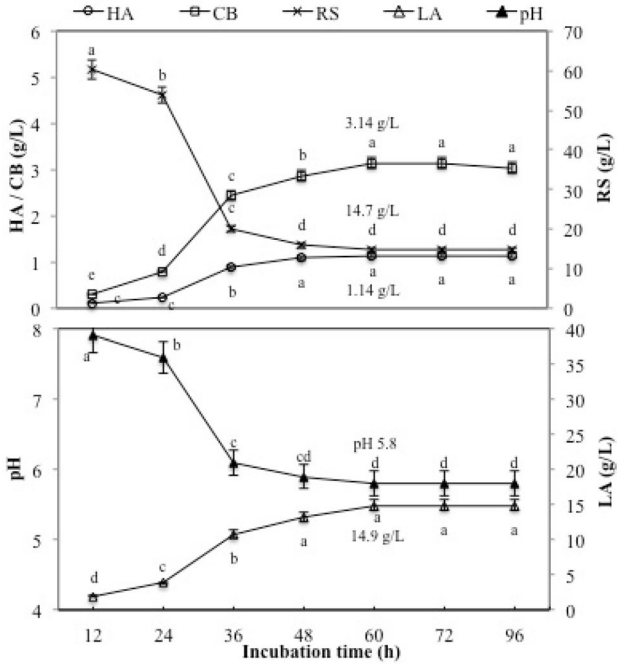


Fig. 4 Time profile of HA, LA and CB production as well as pH change and sugar consumption in molasses medium. The experiments were performed in the medium (control medium) containing only 14% molasses (pH 8.0). *RS* residual sugar, *LA* lactic acid, *HA* hyaluronic acid and *CB* cell biomass. All the measurements were taken in three replicates. Error bars represent standard deviation of replicates. Different lowercase letters in the line of HA, LA, RS, pH or CB indicates significant differences ($P < 0.05$)

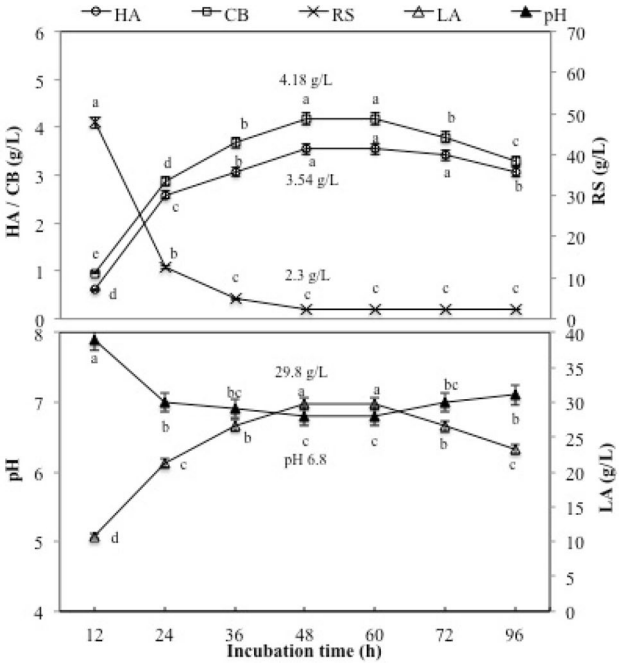


Fig. 5 Time profile of HA, LA and CB production as well as pH change and sugar consumption in SWP-VI medium. The experiments were performed in the medium containing 14% molasses and 12 g/L SWP-VI (pH 8.0). *SWP* sheep wool peptone, *RS* residual sugar, *LA* lactic acid, *HA* hyaluronic acid and *CB* cell biomass. All the measurements were taken in three replicates. Error bars represent standard deviation of replicates. Different lowercase letters in the line of HA, LA, RS, pH or CB indicates significant differences ($P < 0.05$)

faster between 12–24 h of fermentation and continued up to 48th h. In SWP-VI, TP and PP media after 48 h, HA concentrations were 3.54, 2.58 and 2.47 g/L, CB concentrations were 4.18, 3.47 and 3.31 g/L, LA concentrations were 29.8, 32.6 and 35.6 g/L and RS concentrations were 2.3, 4.1 and 3.6 g/L, respectively. The fastest pH drops in SWP-VI, TP and PP media were observed between 12–24 h and the lowest pHs were assayed as respectively 6.8, 5.6 and 5.4 at the end of 48th h. In SWP-VI, TP and PP media, HA, CB, LA and RS concentrations as well as culture pH became stable between 48 and 60 h. After 60th h, HA, CB and LA concentrations decreased in all the peptone media. This decrease might be due to the deficiency of sugar in the media (RS concentrations were only 2.3, 4.1 and 3.6 g/L in SWP-VI, TP and PP media, respectively). Namely, sugar deficiency may have directed the bacterium to use HA as a carbon source. This hypothesis can be supported by the fact that the hyaluronidases synthesized by some *Streptococcus* strains under carbon-limited conditions are able to degrade HA [48–50]. Similarly, the bacterium may have reduced LA content in the medium by using LA as an alternative carbon source due to the low sugar content in the medium. In contrast to the decreases in HA, HB and LA, the culture pH in all the peptone media showed a small increase again after 60th h. This situation could be attributed to the degradation of HA in the medium. The similar result was also reported in a previously published study [51].

Figures 1, 2, 3, 4, 5, 6, and 7 demonstrate that molasses alone could support HA synthesis and cell growth in the bacterium. This result can be explained by the fact that molasses contains some minerals, nitrogenous compounds and B vitamins as well as high sugar content [25, 26, 52]. On the other hand, it was seen that the addition of SWPs and commercial peptones augmented HA and LA synthesis as well as cell growth when compared to the control medium. Furthermore, it was determined that LA and HA synthesis as well as cell growth was faster in the peptone media when compared to the control medium. The positive effect of peptones could be atributed to their chemical compositions, especially their rich protein contents.

The experiments also demosntrated that effect of each peptone on HA and LA synthesis as well as cell growth was different. The best peptone for both HA synthesis and cell growth in *S. zooepidemicus* was determined as SWP-VI followed by TP (Figs. 5, 6, 7). Despite of low protein content of SWP-VI, better HA production in SWP-VI medium was probably due to higher K, P and Mg contents of SWP-VI compared to the

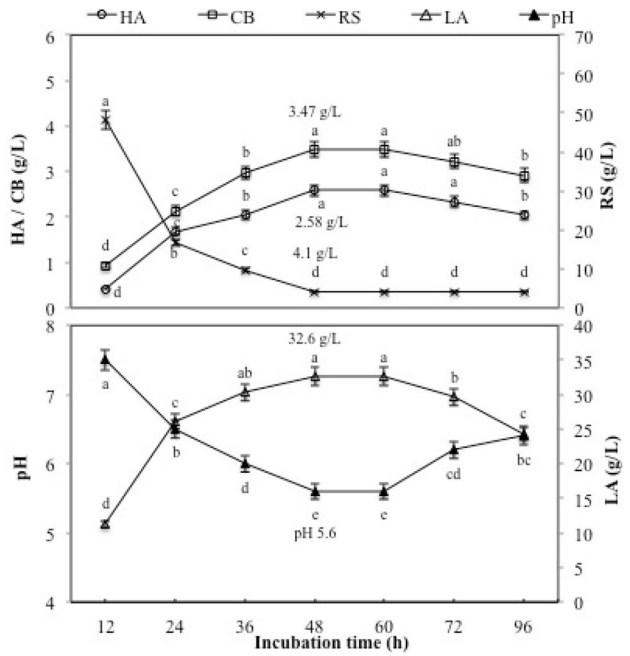


Fig. 6 Time profile of HA, LA and CB production as well as pH change and sugar consumption in TP medium. The experiments were performed in the medium containing 14% molasses and 8 g/L TP (pH 8.0). *TP* trypton peptone, *RS* residual sugar, *LA* lactic acid, *HA* hyaluronic acid and *CB* cell biomass. All the measurements were taken in three replicates. Error bars represent standard deviation of replicates. Different lowercase letters in the line of HA, LA, RS, pH or CB indicates significant differences ($P < 0.05$)

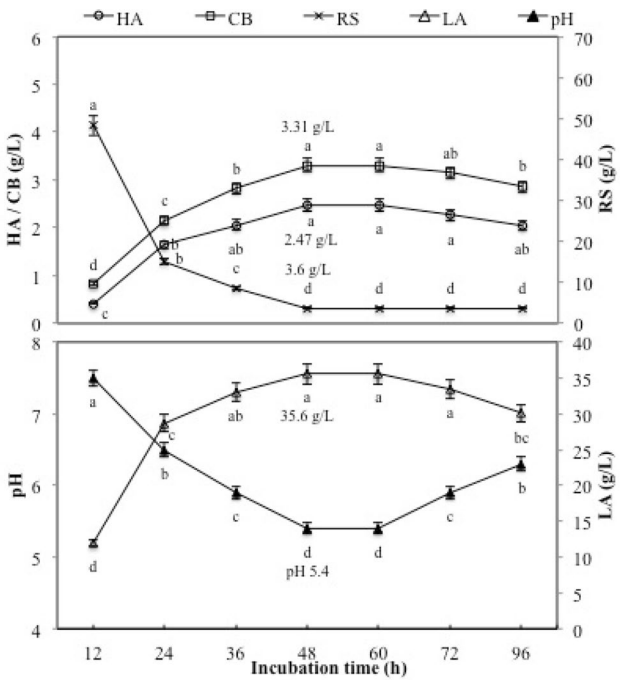


Fig. 7 Time profile of HA, LA and CB production as well as pH change and sugar consumption in PP medium. The experiments were performed in the medium containing 14% molasses and 8 g/L PP (pH 8.0). *PP* protease peptone, *RS* residual sugar, *LA* lactic acid, *HA* hyaluronic acid and *CB* cell biomass. All the measurements were taken in three replicates. Error bars represent standard deviation of replicates. Different lowercase letters in the line of HA, LA, RS, pH or CB indicates significant differences ($P < 0.05$)

commercial peptones (Supplementary Material Table S3). Because, researchers have shown that P, K and Mg are the most important elements that promote HA production [9, 14, 41].

The previous studies have revealed that three most important amino acids for HA synthesis are cystine, lysine and arginine. Especially, arginine has been reported to be main amino-acid that affects HA synthesis in *S. zooepidemicus* [9, 13]. Therefore, it was considered that high cystine and arginine contents of SWP-VI (Supplementary Material Table S4) were the second possible reason of higher HA productivity.

It has been also reported that HA synthesis can be inhibited by excessive LA production and low culture pH. Namely, the acidity in medium directs the carbon source to LA synthesis in *S. zooepidemicus* but the alkalinity does to HA synthesis [53]. Therefore, we assumed that the third reason of higher HA productivity in SWP-VI medium associated with less pH change and/or lower LA synthesis (initial pH and final pH were 8.0 and 6.8 after 48 h, respectively).

Conclusion

Cheap substrate selection in fermentative production of hyaluronic acid (HA) is considered an important criterion. In this context, the present study showed that molasses and sheep wool peptone (SWP-VI) could be used together as cheap complex substrates for fermentative HA production from *S. zooepidemicus*. SWP-VI was found to be more favorable for HA production compared to commercial peptones. Therefore, it can be preferred as a complex substrate (mainly organic nitrogen source) for industrial scale production of HA. The positive effect of SWP-VI on HA production could be attributed to its mineral and/or amino-acid composition. The cost of SWP-VI is lower than the commercial peptones since it can be prepared from sheep wool using cheap chemicals. On the other hand, use of molasses and wool as substrate in HA production may contribute to reducing the environmental pollution problem.

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Compliance with Ethical Standards

Conflict of interest All authors declare that there is no conflict of interest.

Research Involving Human and Animal Rights This article does not contain any studies with human participants or animals performed by any of the authors.

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