Recombinant Production of Hyaluronic Acid

Stephen H. Brown[1] and Philip E. Pummill[2]

[1]*Novozymes, Inc., 1445 Drew Avenue, Davis, CA 95618 and* [2]*Hyalose, L.L.C., 655 Research Parkway, Suite 525, Oklahoma City, OK 73104, USA*

Abstract: Presently, the two main commercial sources of hyaluronic acid (HA) are rooster combs and streptococci. Harvesting from rooster combs is complex and costly. Streptococci are difficult to genetically manipulate and require complex media for growth. Both sources have potential problems with unwanted by-products, such as allergens and toxins. These problems can be solved by producing the HA with safe bacilli that are expressing a recombinant HA synthase (HAS).

Keywords: Hyaluronic Acid, *Bacillus subtilis*, synthase, fermentation.

INTRODUCTION

Hyaluronan (HA) is **synthesized by the polymerization of the monosaccharides from two nucleotide sugars** UDP-glucuronic acid (UDP-GlcUA) and UDP-N-acetylglucosamine (UDP-GlcNAc). The enzyme that catalyzes this reaction is **HA synthase or HAS**. The pathway for HA biosynthesis in the Group A and Group C streptococci has been determined [1,2] (Fig. **1**). Starting from glucose as a carbon source, two parallel metabolic branches can form which eventually, through multiple sugar intermediates, culminate in the synthesis of the two nucleotide sugar substrates, UDP-GlcUA and UDP-GlcNAc. In streptococcal cells, the *hasA* HA synthase gene has been incorporated as the first gene of an operon along with one or more genes (hereafter referred to as "precursor genes") which encode enzymes that are involved in the synthesis of some of the aforementioned precursor sugars. For example, the operons from *S. equisimilis* and *S. zooepidemicus* subsp. *equi* contain four genes: the first, *hasA*, encodes hyaluronan synthase; the second, *hasB*, encodes UDP-Glc dehydrogenase; the third, *hasC*, encodes UDP-Glc pyrophosphorylase; and the fourth, *hasD*, encodes UDP-GlcNAc pyrophosphorylase [3].

The first report on high yield, recombinant production of HA in a GRAS (Generally Recognized As Safe) designation organism was published in 2005 [4]. ***Bacillus subtilis*** was chosen as the expression host for a number of reasons, including a long history of industrial use, and a lack of toxin or hyaluronidase production. In order to produce HA in *B. subtilis*, artificial operons were constructed using the *S. equisimilis* hasA gene [5] and precursor genes from either *S. equisimilis* (*hasB*, *hasC* and *hasD*) or their *B. subtilis* homologues (*tuaD*, *gtaB*, and *gcaD* respectively).

PRODUCTION OF HA IN LAB-SCALE FERMENTORS

One of the strains which produced a pronounced mucoid colony morphology on a plate, RB161, was selected and grown in a 3 liter lab-scale fermentor on a minimal medium with sucrose as the carbon source (Fig. **2**). Samples were collected at various times during the fermentation and measured for HA titer, viscosity, and dissolved oxygen. Maximum production was reached at approximately 25 hours into the fermentation. **HA was produced in the multi-gram per liter range** and compared favorably to published yields obtained from steptococcal fermentations [6]. Due to the viscoelastic properties of HA, the fermentation became very viscous at about 15 hours into the fermentation and concomitantly the dissolved oxygen concentration decreased.

The HA produced in the fermentation was analyzed by GPC-MALLS to determine molecular mass and polydispersity. The *B. subtilis*-derived material consistently measured in the 1.1-1.2 MDa (1,100-1,200 kDa) range with a polydispersity index of 1.5 (*Mw*/*Mn*), comparable to what has been reported for commercially available sources [7].

UDP-GLUCURONIC ACID IS LIMITING FOR HA SYNTHESIS IN *B. SUBTILIS*

Significant amounts of HA are produced by strain RB161. Besides the *hasA* gene, this strain has been engineered to overexpress the *tuaD* and *gtaB* genes as well, which are involved in the synthesis of UDP-GlcUA and UDP-Glc, respectively. In order to determine if either of these UDP-sugars is limiting for HA production, strains RB182 and RB184 were constructed; these strains overexpress *hasA* alone or *hasA* and *tuaD*, respectively. Both strains were grown in fermentors and analyzed for HA production (Fig. **3**). Yields from RB182 were negligible, whereas yields from RB184 were comparable to strain RB161 indicating that **the level of UDP-GlcUA is limiting in the native *B. subtilis* background** due to insufficient levels of UDP-Glc dehydrogenase. This observation is actually expected in that the native *tuaD* gene is reported to be repressed by phosphate, which is in excess during these fermentations [8].

Overexpression of the precursor genes *hasB* and *hasC* is expected to increase the levels of UDP-GlcUA in the cell. We postulated that increasing the levels of the other sugar substrate, UDP-GlcNAc, could also have a positive impact on HA production. The *hasA* operon from *S. equisimilis* does

*Address correspondence to this author at the Novozymes, Inc., 1445 Drew Avenue, Davis, CA 95618 and [2]Hyalose, L.L.C., 655 Research Parkway, Suite 525, Oklahoma City, OK 73104; E-mail: ppummill@hyalose.com

1389-2010/08 $55.00+.00

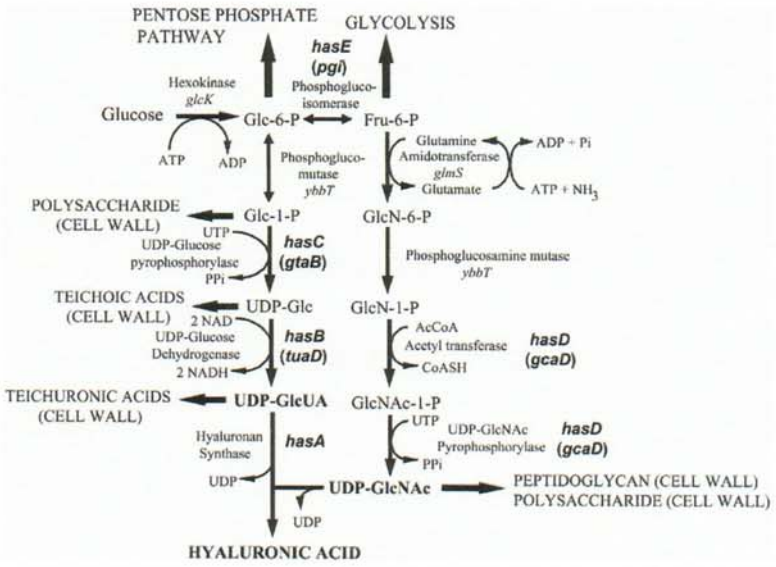

Fig. (1). Proposed biosynthetic pathway for HA production in recombinant *B. subtilis* strains. This pathway is based on the reported HA biosynthetic pathway in Group A and C *Streptococcus* (1, 2), homology searches against the published *B. subtilis* genome sequence, and the *B. subtilis* metabolic pathways described in the Kyoto Encyclopedia of Genes and Genomes (http://bioinformatics.weizmann.ac.il:3456/-kegg/kegg.html). Gene designations are highlighted in boldface. The *B. subtilis* homologs of streptococcal *has* genes are in parentheses.

contain a fourth gene, *hasD*, which encodes UDP-GlcNAc pyrophosphorylase. This bifunctional enzyme catalyzes the last two steps in the synthesis of UDP-GlcNAc. To test whether overproducing this particular enzyme could further boost HA yields, strain RB163 was constructed. This strain overexpresses *gcaD*, the *B. subtilis* homologue to *hasD*, in addition to *hasA* and *tuaD*. Fermentation results from this strain show that HA yields are essentially equal to those obtained from RB161 and RB184 (Fig. **3**). Similar results were obtained from a *B. subtilis* strain expressing the *S. equisimilis* operon: *hasA*/*hasB*/*hasC*/*hasD* (data not shown). These results could indicate that HA production by RB161 and RB184 is not limited by UDP-GlcNAc or perhaps if they are, the limiting step is earlier in the biosynthetic pathway than the step catalyzed by UDP-GlcNAc pyrophosphorylase, which leads to this precursor sugar.

DISCUSSION

B. subtilis has proven to be a superior expression host for producing HA based on several criteria. The yield and quality of the product are very good, both with regards to molecular mass and polydispersity. In addition, the *B. subtilis*-derived HA is exotoxin-free which can be problematic in certain streptococcal strains. The HA is secreted into the surrounding media and is not cell-associated which should greatly simplify the recovery process and facilitate downstream processing. The production economics are further

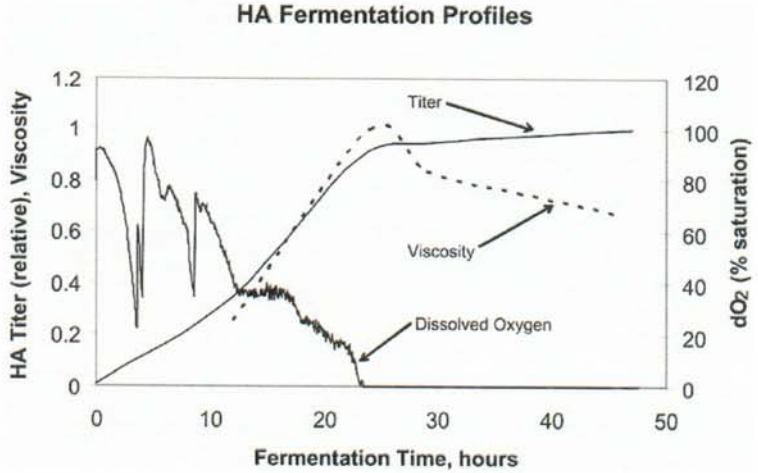

Fig. (2). Fermentation profiles of strain RB161 showing relative levels of HA produced, viscosity, and dissolved oxygen as a function of time.

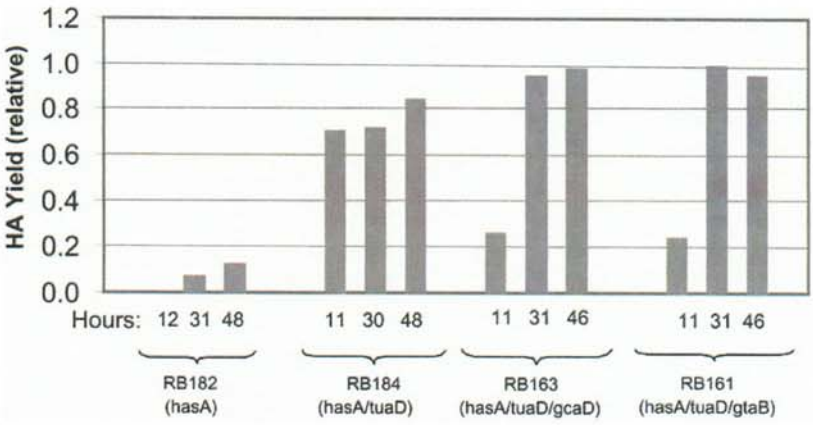

Fig. (3). Effect of precursor gene overexpression on HA productivity in *B. subtilis*. The HA content of a medium sample, at the indicated time, was determined. The genes in the artificial *has* operons of the indicated strains are in parenthesis.

enhanced due to the ability of *B. subtilis* to grow on minimal media, in contrast to the Streptococci Group A and C which are fastidious organisms and require more expensive complex media for growth.

In *B. subtilis*, we have shown that the levels of UDP-GlcUA are limiting with regards to high-level HA production, at least when grown in the presence of excess phosphate. This was shown conclusively by comparing the *hasA* versus the *hasA/tuaD*-overexpressing strains. It is interesting to note, that overexpressing *hasA* alone does not seem to hinder cell growth, *i.e.* the hyaluronan synthase is not titrating away sugars that are required for cell growth, at least not to dangerously low levels. This is in contrast to *E. coli*, where overexpression of *hasA* by itself severely compromises the cells ability to grow [9]; this effect can be avoided if a strain lacking the *ugd* gene (a *tuaD* homologue) is utilized. Evidently in *B. subtilis* this is not a problem as evidenced by the *hasA/tuaD* overexpressor in which high-level production of HA and cell growth are supported simultaneously. Clearly *B. subtilis* is inherently more metabolically robust than *E. coli*, at least with regards to HA production.

Considering the ease with which *B. subtilis* can be manipulated, this expression system could lend itself as **an ideal model system for dissecting the complex enzymology of hyaluronan synthase**. Even though it is a small protein, this enzyme must perform multiple functions in order to polymerize the two sugars and transport the growing HA polymer to the outside of the cell; no less than seven distinct functions have been proposed [10]. Structure/function studies have yielded valuable bits of information concerning HAS topology and organization within the membrane, but so far they have been limited to low numbers of site-directed mutants and measures of enzyme function have been limited to *in vitro* assays [11]. It would be advantageous to have a system whereby large numbers of engineered HAS variants could easily be generated and screened *in vivo* for activity. Technically, this would be extremely challenging with a Streptococcal-based system due to the difficulties and inefficiencies in genetically manipulating this organism. However, a *B. subtilis*-based expression system should be very amenable to this sort of analysis. Ultimately, a more detailed understanding of HA synthesis could lead to custom-tailored products, such as targeted molecular weight ranges, some of which will undoubtedly result in the development of new and exciting applications for this versatile product in the future.

REFERENCES

[1] Matsubara, C.; Kajiwara, M.; Akasaka, H. and Haze, S. (1991) *Chem. Pharm. Bull.*, **39**(9), 2446-2448.
[2] O'Regan, M.; Martini, I.; Crescenzi, F.; De Luca, C. and Lansing, M. (1994) *Int. J. Biol. Macromol.*, **16**(6), 283-286.
[3] Sloma, A.; Behr, R.; Widner, W.; Tang, M.; Sternberg, D. and Brown, S. (2003) World Patent Application WO03/054163
[4] Widner, B.; Behr, R.; Von Dollen, S.; Tang, M.; Heu, T.; Sloma, A.; Sternberg, D.; Deangelis, P.L.; Weigel, P.H. and Brown, S. (2005) *Appl. Environ. Microbiol.*, **71**(7), 3747-3752.
[5] Kumari, K. and Weigel, P.H. (1997) *J. Biol. Chem.*, **272**(51), 32539-32546.
[6] Kim, J.-H.; Yoo, S.-J.; Oh, D.-K.; Kweon, Y.-G.; Park, D.-W.; Lee, C.-H. and Gil, G.-H. (1996) *Enzyme Microb. Technol.*, **19**(6), 440-445.
[7] Adam, N. and Ghosh, P. (2001) *Inflamm. Res.*, **50**(6), 294-299.
[8] Soldo, B.; Lazarevic, V.; Pagni, M. and Karamata, D. (1999) *Mol. Microbiol.*, **31**(3), 795-805.
[9] DeAngelis, P.L.; Papaconstantinou, J. and Weigel, P.H. (1993) *J. Biol. Chem.*, **268**(26), 19181-19184.
[10] Weigel, P.H. (2002) *IUBMB Life*, **54**(4), 201-211.
[11] Heldermon, C.D.; Tlapak-Simmons, V.L.; Baggenstoss, B.A. and Weigel, P.H. (2001) *Glycobiology*, **11**(12), 1017-1024.