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Optimization of cloning and expression of 6His-tagged 196 aa length oncostatin M in *E. coli*

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A short, active form of oncostatin M protein consists of 196 aa and is 23 kDa protein. Using a full length open reading frame of this protein in pCMV type shuttle a PCR was performed and optimized in order to obtain the required form. A 6His tag was also added. As this active protein form is toxic to the most of the cells, accurate optimization of biosynthesis is required. A potent pT7a plasmid with double lacO sequence and early bacteriophage T7 promoter ensured highly stringent, controllable expression and its levels are further raised using medium optimization.

Key words: oncostatin M, PCR, biosynthesis, SDS-PAGE, bioreactor

INTRODUCTION

Oncostatin M (OSM) is a cytokine that acts in diverse biological activities, including gene expression in acute inflammation phase, antitumor activity and cell growth modulation. This cytokine is most commonly grouped with similar proteins with the same activities, such as IL-6, LIF, CNTF and others. This protein is of great medical potential as it has been used to slow down cancer varieties, regulating inflammation processes, synergistically with interferons treating chronic hepatitis C. However, as its main activity is to slow down the growth of the cells, recombinant production of this protein can be difficult. In this article we present an optimized scheme for cloning, transformation and expression of active form of OSM with 6His-tagged N-terminal end. It must be explained that mature mRNA of OSM produces a 252 aa length protein which has two sequences that are proteolitically cleaved during maturation of the protein. A 25 aa N-terminal leader sequence is cleaved leaving a 227 aa length OSM pro-sequence which is 8-fold less active than the circulating 196 aa length form. In the current market, a 227 aa length protein form is dominating, due to its slower inhibiting activity. Therefore, it is a constant need of both tagged and untagged OSM(196) form. As several articles exist concerning OSM(196) production (Sporeno et al., 1994; Kong et al., 2009) there are few attempts to optimize biosynthesis in *E. coli* in bioreactor.

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MATERIALS AND METHODS

The full length sequence of OSM gene (including both pre- and prosequences) was purchased from Origene™, in a pCMV DNA shuttle. The sequence was PCR amplified using complement primers to 26-221 aa sequence.

The pT7 plasmid used for cloning contains various genetic elements, such as kanamycin resistance gene, ori site for plasmid replication, lacI gene for lac operon repression, multiple cloning site and an early phage T7 promoter with ribosome binding site and double lacO sequence repetition following it. This plasmid is known to maintain a stable and high expression of middle-sized recombinant proteins in various lac based bacterial producents. The most convenient way to insert gene into expression cassette is via *NdeI* site which includes ATG start codon and *HindIII*, XhoI or BamHI site in the end of gene. The promoter sequence used in this plasmid is early promoter from bacterial phage T7. This sequence is known for its strong and stringent control of protein expression. Accompanied with lacO operator sequence, this promoter is suitable for induction of recombinant protein expression with IPTG and can be used with T7 DNA polymerase based bacterial systems (such as *E. coli* BL21(DE3), *E. coli* K802 and others).

Three media for cell cultivation were used, namely standard LB, M9 and modified M9m. Composition of each medium is specified: LB medium (for inoculum) composition (g/L): yeast extract – 5, tryptone – 10, sodium chloride – 10. M9 medium (for fermentation) composition (g/L): yeast extract – 20.0, ammonium chloride – 5.0, magnesium sulfate heptahydrate – 0.5, di-sodium hydrogen phosphate – 4.7, potassium dihydrogen phosphate – 4.5, glucose monohydrate – 22. M9m medium (for inoculum) composition (g/L): yeast extract – 10, ammonium chloride – 1, magnesium sulfate heptahydrate – 0.5, di-sodium hydrogen phosphate – 4.7, potassium dihydrogen phosphate – 4.5, glucose monohydrat – 22. M9m medium (for fermentation) composition (g/L): yeast extract – 20.0, ammonium chloride – 5.0, magnesium sulfate heptahydrate – 0.5, di-sodium hydrogen phosphate – 4.7, potassium dihydrogen phosphate – 4.5, glucose monohydrate – 22.

The samples were analysed in 4–15% SDS-PAGE gel in denaturing conditions and Western-blotted on PVDF membrane using a mAb against OSM (Abcam, cat. No. ab-10842, Ms Mab to Oncostatin M [17001.31]), paired with secondary antibody against mouse IgG (ECL Anti-Mouse IgG, Horseradish Peroxidase linked F(ab')2 fragment from sheep, GE Healthcare, cat. No. NA9310V) and visualised with 3, 3', 5, 5' tetramethylbenzidine substrate.

RESULTS AND DISCUSSION

Cloning of 6His-tagged OSM(196)

Cloning of 6His-tagged OSM(196) consisted of adding 18 base pairs coding for six histidine residues to the 5' end of gene (to the N-end of protein accordingly). This process was done by executing a PCR amplification with flanking forward PCR primer.

Primers were as presented in Table 1, adding six histidine residues codons and *NdeI* recognition site on the 5' gene end and *HindIII* recognition site on the 3' end. Three and four base pairs of T-A nucleotides were added behind both recognition sites to ensure proper digestion of PCR product with restriction endonucleases.

The underlined sequences are *NdeI* recognition and cleavage site for forward primer (*CA^TATG)* and *HindIII* for reverse (*A^AGCTT*). Bold sequence is 18 bases coding for six histidines in front of start alanine:

PCR optimization for His-tagged OSM(196) was performed in order to receive a specifically amplified PCR product in desirable concentration interval. Optimisation parameters included

Table 1. Primer sequences and their main features for PCR amplification

Primer	Sequence (5'→3')	Restriction endonuclease site	Tm
FOR-OSM-6HIS:	TTAACATATG**CACCACCACCACCACC AC**GCGGCTATAGGCAGCTGCT	*NdeI* (CATATG)	80.1 °C
RSRR-REV-HIND	TTTAAGCTTCTATCTCCGGCTCCGGTT	*HindIII* (AAGCTT)	65.1 °C

$MgSO_4$ concentration (1–4 mM), primers concentration (0.1–1 μM) and annealing temperature (63–72 °C), thus forming a net experiment of 48 different reaction conditions. DNA polymerase used for amplification was *Pfu* DNA polymerase (recombinant, Fermentas, Thermo Fisher Scientific) due to its proof-reading feature, good processivity and A low error rate.

For this particular PCR product, the best conditions were as follows: 3 mM of $MgSO_4$, 1 μM of each primer, and program with double annealing steps:

95 °C	**95 °C**	**68 °C**	**72 °C**	**95 °C**	**72 °C**	**72 °C**
3’	30”	45”	80”	30”	80”	10’
		15×	Go to #2	20×	Go to #5	

This strategy was chosen due to very different melting temperatures of primers. The temperatures could not have been normalized because of the flanking primer strategy. The PCR product was attainable in 100–120 ng/μl concentration range.

The obtained and purified PCR product was double-digested with *NdeI* and *HindIII* restriction endonucleases, shedding 6–8 base pairs from each gene end (see Fig. 1 for full scheme of cloning). These residues were removed with PCR Purification Kit (Thermo Fisher Scientific, Fermentas) and the sample was concentrated twice as regarding its starting volume. The prepared digested gene was ligated into respectively prepared pT7a plasmid, which was additionally dephosphorylated (with recombinant CIAP, Thermo Fisher Scientific, Fermentas) to reduce self-ligation clones background.

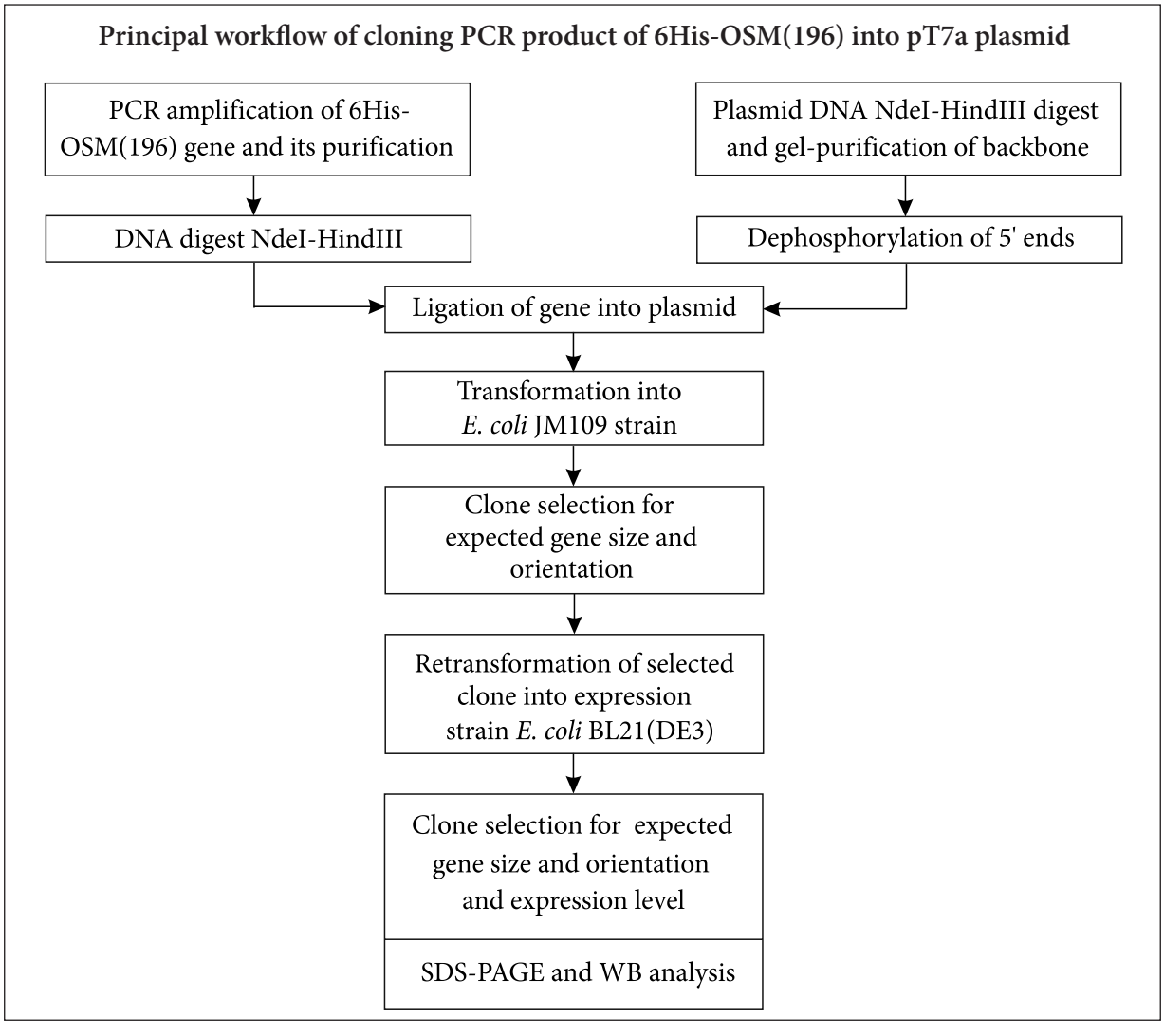

Fig. 1. Principal workflow scheme of cloning PCR product

Dephosphorylated plasmid was additionally purified loading it on agarose gel, excising backbone fragment from gel and purifying it with GeneJet Gel Purification Kit.

Clone selection and restriction analysis of 6His-OSM(196)/pT7a construct

NdeI-HindIII digested PCR product was ligated into a pT7a plasmid with the molar gene to plasmid ratio of 10:1. The mixture then was transformed into *E. coli* JM109 strain using Fermentas TransformAid Kit. The JM109 strain used in first steps of cloning is stable gyrase- and endonuclease-free strain which enables safe cloning of ligated constructs. The clones obtained after plating competent cells with gene-plasmid ligation mix onto Petri dishes with 50 mg/L kanamycin were checked for the insert size by digesting plasmid DNA with *NdeI* and *HindIII*. The resulting DNA fragments for positive clones were 5 300 bp (plasmid backbone) and 606 bp (gene insert). Plasmid DNA from selected correct clones was extracted and full restriction analysis was made (see Fig. 2). Restriction map and fragment length table are presented below and were used whenever recloning was done. After the clones subjected to analysis showed full consistency to their theoretical restriction analysis, selected plasmid DNA was retransformed into *E. coli* BL21(DE3) strain. Again, after transformation both double digestion with *NdeI* and *HindIII* and full restriction analysis were performed, and clones then were selected for their expression rates and plasmid stabilities.

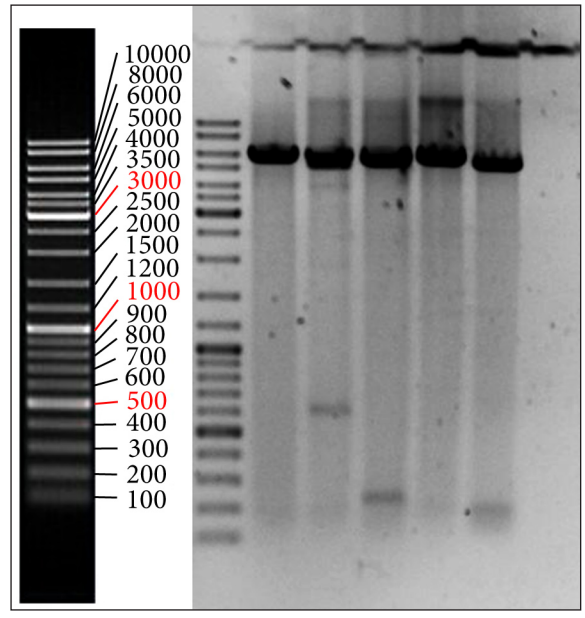

Fig. 2. Restriction analysis of the 6His-OSM(196)/pT7a

Finally, one clone containing pT7a plasmid matching the expected structure (Figs. 2 and 3) and restriction pattern (Table 2) has been selected for sequencing. The insert of 6His-OSM(196) from the plasmid pT7a was sequenced using Applied Biosystems 3 130 × l Genetic Analyzer. The primers used for sequencing were fully complementary to the insert and sequenced and merged sequence was 100% complement to theoretical.

Biosynthesis of selected 6His-OSM(196)/pT7a clone in flasks

Selected clone of 6His-OSM(196)/pT7a/*E. coli* BL21(DE3) was inoculated from overnight cul-

Table 2. Restriction pattern of 6His-OSM(196) pT7a constructs

Number	Restriction endonuclease	Theoretical fragment size, bp	Practical fragment size, bp
1	EcoRV	5 889	6 000
2	*NdeI-HindIII*	5 283	5 500
		606	600
3	*NdeI*-BglII	5 619	5 600
		200	200
		70*	
4	Bsu36I	5 889	6 000
5	PstI	5 509	5 500
		170	180
		120	100
		*90	

* – the fragments that are poorly visible in agarose gels

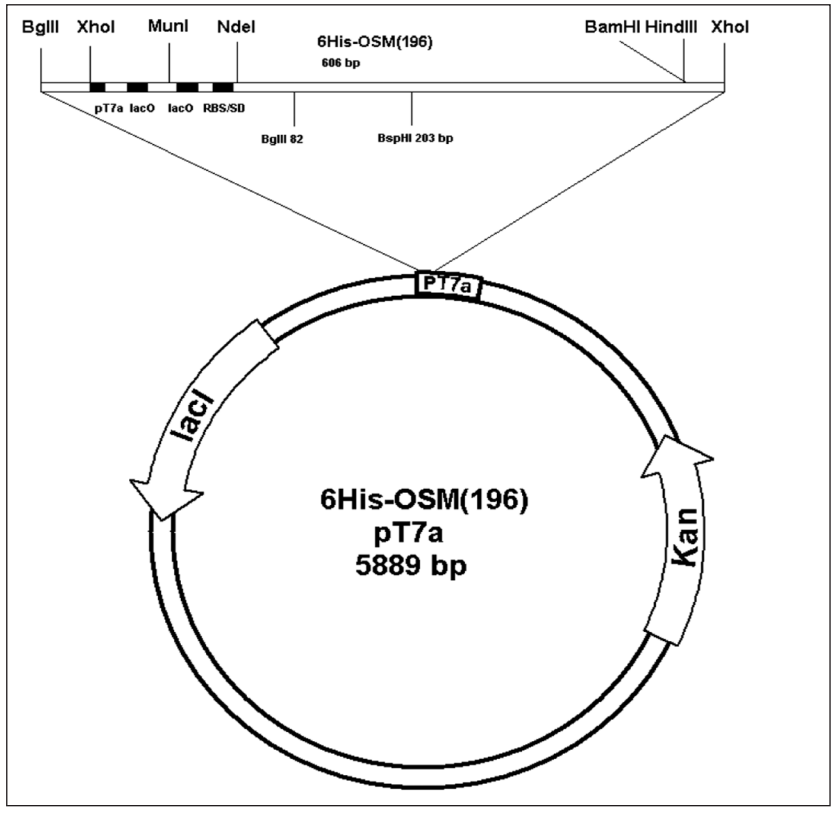

Fig. 3. Restriction map of 6His-OSM(196)/pT7a

ture in 2 flasks containing 500 ml of LB medium with 50 mg/L kanamycin. Starting optical density for both flasks was 0.2 optical units. Flasks were cultivated at +37 °C, 200 rpm in microbial orbital shaker. After two hours following the inoculation, one flask was induced with standard 0.5 mM IPTG while the density reaching 1.0 optical unit, and the second flask was used to propagate in the same conditions as a non-induced reference. After 3 hours after induction, all biomass was harvested and weighed, resulting in 1.0 g of induced culture and 1.6 g of non-induced culture. In Fig. 4 a graph of growth curves in both flasks is presented.

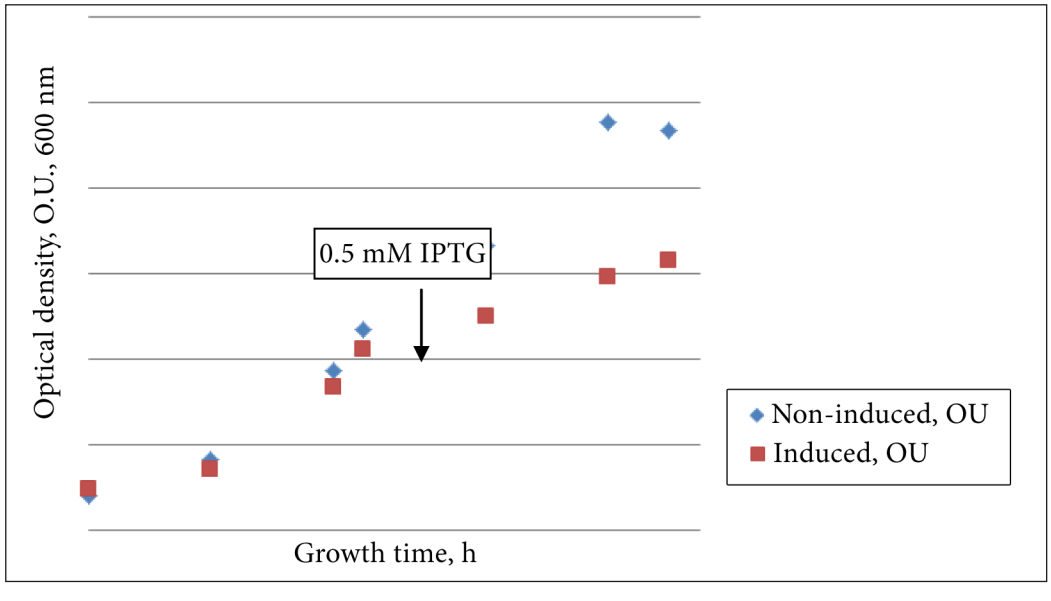

Fig. 4. Growth curves of 6His-OSM(196)/pT7a/*E. coli* BL21(DE3 in 500 ml LB medium flasks

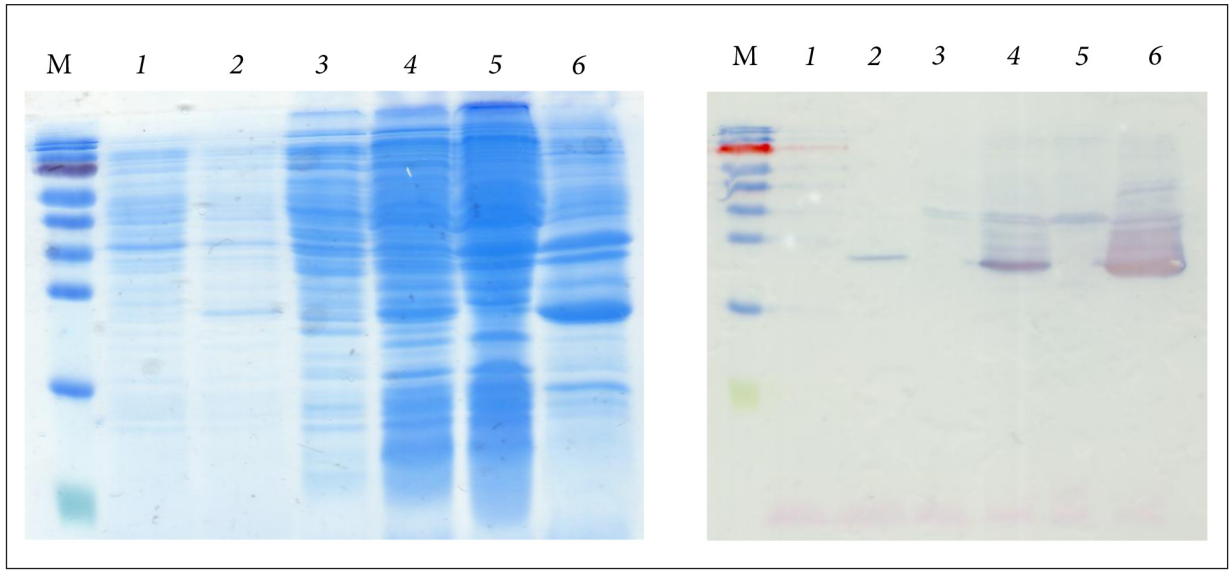

Fig. 5. SDS-PAGE and WB of 6His-OSM(196)/pT7a/*E. coli* BL21(DE3)

Lanes: *1* – non-induced culture, sample of total protein fraction, *2* – induced culture, sample of total protein fraction, *3* – non-induced culture, soluble fraction, *4* – induced culture, soluble fraction, *5* – non-induced culture, insoluble fraction, *6* – induced culture, insoluble fraction

Expression analysis for 6His-OSM(196) in flasks

A harvested culture from the above described flask cultivation was suspended in 10 and 16 ml of resuspension buffer (100 mM Tris-HCl, pH 8.0 containing 5 mM EDTA, 0.1% Triton, 0.1% PMSF) for induced and non-induced cultures, respectively. The cell suspension was disintegrated using Sonics VibraCell ultrasonic disintegrator, with impulses of 1 second – on, 2 seconds – off, with 40% power. Soluble fraction was separated from insoluble inclusion bodies centrifuging the disintegrated solutions for 20 minutes in microcentrifuge at 12 000 g.

The samples were analysed in 4–15% SDS-PAGE gel in denaturing conditions (as presented in Fig. 5) and Western-blotted on PVDF membrane. As it may be seen, in all samples of induced culture a 23 kDa-size protein is visible.

Biosynthesis of 6His-OSM(196)/pT7a in the fermentor

In all further studied cases batch mode biosynthesis of *E. coli* BL21(DE3)/pT7a/6His-OSM(196) were performed in 2 L working volume fermentor "Biostat M" at pH 6.8–7.0, pO_2 – 30% with automatically controlled on-line variables (temperature, stirring, pH, pO_2) and off-line variable – optical density.

Initially, for comparison of developed strain-producer *E. coli* BL21(DE3)/pT7a/6His-OSM(196) productivity, 2 L batch fermentations using M9 and M9m media were performed . After 3 h of culture growth the biosynthesis of the target protein was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). As data presented in Table 3 and Figs. 6 and 7 show, a slightly higher productivity of protein biosynthesis process was observed using M9m media. As shown in Fig. 5, the target protein is accumulated within the cells of *E. coli* BL21(DE3) pET21a+/6His-OSM196 as inclusion bodies and is recognized in Western blot analysis by antibodies specific to His-tag and antibodies specific to oncostatin M.

Plasmid stability before induction by replica plating in all cases was not less than 100%; after induction – not less than 65%.

As it can be seen medium change from LB to rich type of M9 allows us to achieve up to 3.5-fold optical density enrichment. Comparison between modified and normal M9 media shows 2-fold increment of recombinant protein yield. Plasmid stabilities during M9m fermentation were also more satisfying (data not shown). Fig. 8 includes results from Fig. 7 and Table 3, recalculating recombinant protein yield according to biomass accumulation in volume of fermentation suspension.

Table 3. Biosynthesis parameters and productivity of the strain-producer *E. coli* BL21(DE3)/pT7a/6His-OSM(196) with M9 and M9m media

Controlled parameters	M9 medium	M9 modified medium
Working volume, L	1.5	1.4
Final IPTG, mM	0.5	0.5
Induction time, h	3	3
Induction at OD	2.3	2.7
Final OD	3.8	5
Yield of wet biomass, g/L	7.2	10.9
Total proteins, mg/g wet biomass	61.4	79.7
6His-OSM196, % from total proteins (SDS-PAGE)	17–19	22–26
6His-OSM196, mg/g biomass (calculated acc. total proteins)	10.4–11.6	17.5–20.7
6His-OSM196, mg/L cell suspension (acc. total proteins)	75–84	191–226

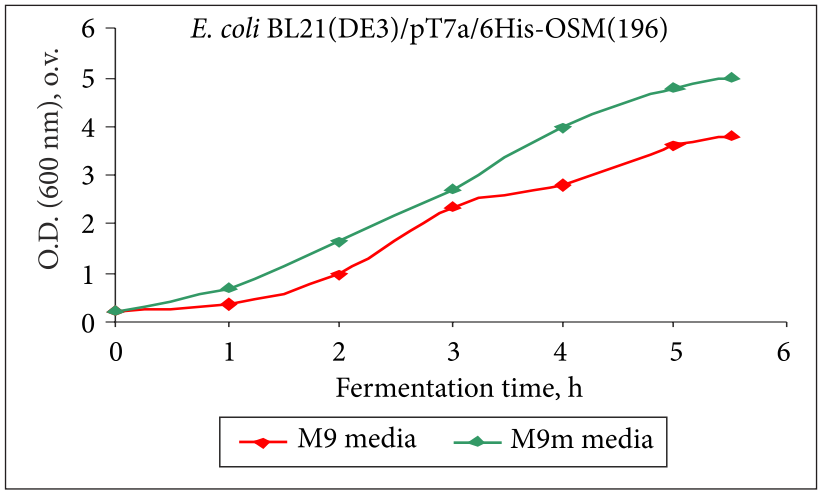

Fig. 6. Growing of *E. coli* BL21 (DE3) pT7a/6His-OSM(196) with M9 and M9m media

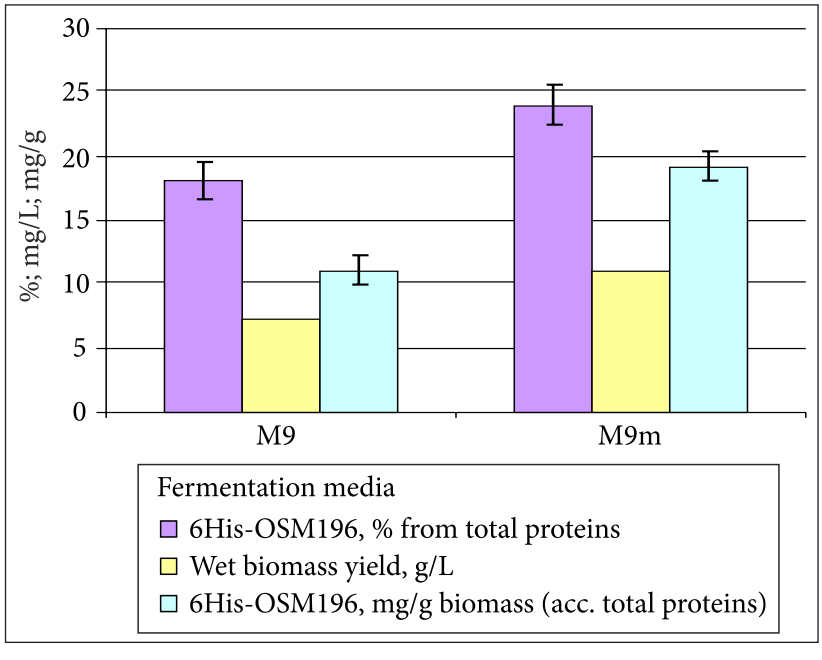

Fig. 7. Biomass production and yield of target protein at *E. coli* BL21 (DE3)/pET21a+/6His-OSM 196 biosynthesis with M9 and M9m media

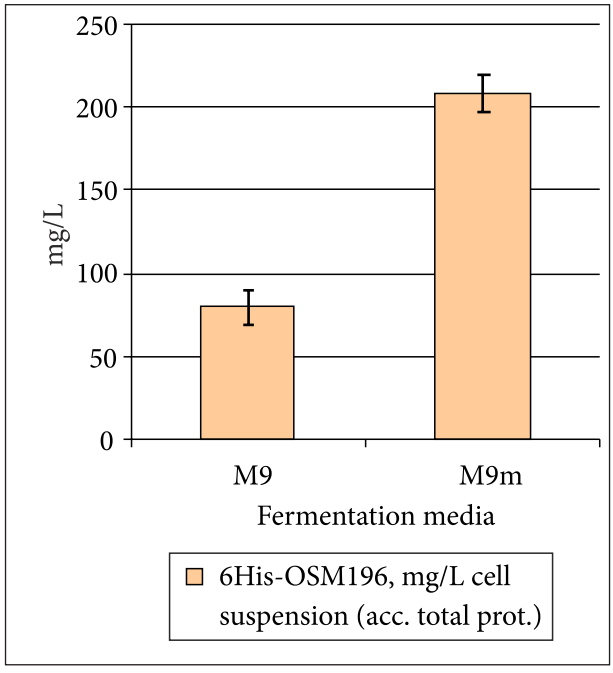

Fig. 8. *E. coli* BL21(DE3)/pET21a+/6His-OSM196 productivity at biosynthesis with M9 and M9m media

CONCLUSIONS

Even though a lot of experiments are performed in order to obtain either of OSM forms, with or without histidine tags, very little is known or optimized for OSM(196) form and its production in fermentor scale. The use of an early bacteriophage T7 promoter and double lacO sequence upstream enabled to obtain a highly productive, stringently controllable expression of recombinant gene. The optimization of the medium results in 3-fold increment of biomass yield, and 2-fold increase of recombinant protein yield.

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196-ių AMINORŪGŠČIŲ ILGIO ONKOSTATINO M SU 6HIS INKARINE SEKA KLONAVIMO IR GENO RAIŠKOS *E. COLI* OPTIMIZAVIMAS

Santrauka

Trumpoji ir biologiškai aktyvi onkostatino M baltymo forma susideda iš 196 aminorūgščių ir yra 23 kDa molekulinio svorio. Naudojant visą atviro skaitymo rėmelį iš pCMV nešančiosios plazmidės buvo atlikta PGR reakcija bei jos optimizavimas, kad būtų gauta reikiama forma, taip pat buvo pridėta 6His inkarinė seka. Kadangi aktyvus baltymas yra toksiškas daugumai ląstelių, būtinas kruopštus biosintezės optimizavimas. Galinga pT7a plazmidė su dviguba lacO seka ir ankstyvu bakteriofago T7 promotoriumi užtikrino griežtą, kontroliuojamą geno raišką, kurios lygis toliau pagerinamas optimizuojant bakterijų auginimo terpę.

Raktažodžiai: onkostatinas M, PGR (polimerazės grandininė reakcija), biosintezė, NDS-PAAG (natrio dodecilsulfato poliakrilamidinio gelio elektroforezė), bioreaktorius