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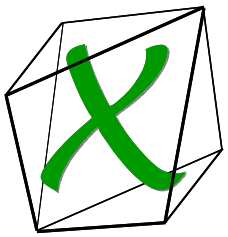

Making Se-Met proteins in non-auxotrophic *E.coli*

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This protocol is based on the original method of S. Doublié (Met. Mol. Bio 363-vol.1 p.91). It has been modified to make the process more streamlined and to increase the yield of biomass and (hopefully) of the purified labeled protein. This protocol is written for folks who are intimately familiar with the essentials of *E. coli* culture. It is also assumed that the conditions for optimum expression of the target protein have been established (and that rich medium is not detrimental to expression of the target).

Prior to experiment: Prepare a mixture of essential organic components. It is often convenient to prepare this mixture in aliquots suitable for the production of 6L batches of media (Table 1). Each aliquot fits into a 50-ml Falcon tube and once mixed may be stored indefinitely at 4ºC. This is by far the most time-consuming and boring step of the entire process.

Late afternoon on the day before: start an overnight culture of your production strain in 50-200 ml of unlabeled rich medium (such as TBGG[*]) supplemented with appropriate antibiotic(s). Try to time your growth so that you can expect late log phase by morning of the next day (lower growth temperature to slow the culture if necessary). Obtain or prepare 1 liter of sterile unlabeled rich medium (TBGG). Prepare sterile glassware and water for the main fermentation, gather all the remaining reagents.

Morning of the experiment: transfer some (or all) of the overnight culture into 1 liter of pre-warmed unlabeled rich medium, supplemented with the requisite antibiotic(s). Grow this culture at 37ºC with good aeration so that it doubles rapidly. It is convenient to time the growth so that you have a couple of hours to do the rest of the work – therefore use enough of the starter culture to give you about 1 OD_{600} starting cell density, which should become 6-10 OD_{600} in about two hours.

While the 1L culture is growing: resuspend the essential organic components in a convenient volume of water (e.g. 900 ml). Stir the mixture vigorously to break down all the clumps. Distribute the resulting suspension evenly into six 1L glass baffle flasks (or other fermentation vessels of your choice). Add water to each flask so that the final volume is ~1L. Add the remaining ingredients to each flask: trace minerals, vitamin solution, salts, glucose, glycerol (Table 2 and Table 3) Final pH should be 7.0-7.4 – adjust with NaOH as needed. Don't forget to add the antibiotic(s) of choice.

[*] TBGG: "Terrific Broth" (TB) supplemented with 0.8-1.0% glucose and 3-4% glycerol

Finally add the seleno-methionine as powder. In our experience 85-130mg of L-SeMet per liter of medium works well. If you use racemic Se-Met you have to double its amount – please note that selenium is moderately toxic to E. coli and therefore Se-Met concentrations in excess of 180 mg/L should be avoided. Note that some of the components of the complete Se-medium may not be fully in solution – this is not detrimental to the outcome of the fermentation, as these components gradually dissolve during fermentation. It often helps to place the flasks in the 37C shaker for 30 minutes prior to inoculation.

It is prudent to use sterile-filtered water and sterilized glassware (especially for overnight inductions) however it is not necessary to use sterile technique at this point; nor is it adviseabl to filter-sterilize the complete Se-medium. You will be inoculating the Se-medium at such high cell densities that any competing microorganisms do not stand a chance (unless they're in the starter inoculum to begin with).

While the 1L culture is ready: Harvest the 1L unlabeled culture when it is at 11-12 OD_{600} (the use of sterile plasticware is welcome but not essential at this point). Spin the culture for ~10-15 minutes at 4000-5000 rpm in a refrigerated centrifuge (typically the medium will cool to about 10ºC if the centrifuge is set for 4ºC). Gently discard the supernatant, and immediately resuspend the pellet in a convenient amount of warm Se-medium. Distribute the resuspended pellet evenly across the six fermentation flasks (may be pre-warmed). Note the starting optical density of the culture (which should be ~2 OD_{600}). Incubate the culture for ~90 minutes at 37ºC. Check the optical density – it should be the same or slightly higher than it was upon inoculation. If the cell density decreased, allow the culture to recover for an additional hour. At this point you should induce your culture in the manner that you have worked out for the unlabeled fermentation; change the growth temperature to the optimum value; and incubate the culture for the required amount of time. As a rule of thumb it is useful to add 30-50% to the induction time to compensate for the effects of defined medium and selenium.

After the fermentation is complete: measure the final optical density – if your culture grew reasonably well you may get 8 OD_{600} or more. Harvest the cells in the usual manner (e.g. 5000 rpm for 20 minutes at 4ºC). Note that the cell pellet will contain a small amount of inorganic precipitate that resembles fine sand in appearance. This precipitate is likely composed of selenate/ selenite salts. Also note that the spent medium should normally be treated as toxic waste and disposed of in an appropriate manner.

As the traditional Ukrainian toast goes:

"May your bugs grow fat and happy!"

Table 1. Basic organic components of the Se- medium (in grams):

	For 1L of culture	for 6L of culture
Cys	0.2	1.2
His	0.2	1.2
Ala	0.3	1.8
Arg*HCl	0.3	1.8
Asp	0.3	1.8
Asn	0.3	1.8
Glu	0.3	1.8
Gln	0.3	1.8
Gly	0.3	1.8
Leu	0.3	1.8
Pro	0.3	1.8
Ser	0.3	1.8
Trp	0.3	1.8
Tyr	0.3	1.8
Thr	0.3	1.8
Phe	0.3	1.8
Lys*HCl	0.3	1.8
Ile	0.3	1.8
Val	0.3	1.8
Adenine	0.5	3
Cytidine	0.5	3
Guanine	0.5	3
Thymine	0.5	3
Uracil	0.5	3
Thiamine	0.015	0.09
Biotin	0.015	0.09
Pyridoxine	0.015	0.09

Table 2 – other solid ingredients (in grams):

	For 1L of culture	for 6L of culture
NaOAc	1.5	9
Succinic acid	1.5	9
NH_4Cl	0.8	4.8
Na_2HPO_4	6.0	24
KH_2PO_4	3.0	18
Glucose	8.0	48

Table 3 – additional liquid ingredients (in ml):

	For 1L of culture
Glycerol	35
1M $MgSO_4$	2
100 mM $CaCl_2$	1
Kao&Michayluk vitamins	10**
1000x trace element mix	1

Final pH should be 7.0-7.4 – adjust with NaOH as needed.

** This ingredient may be omitted if you don't have the solution handy.

Appendix

Trace-element mix (1000X) in mg/L

$ZnSO_4*7H_2O$	4400
$MnCl_2*4H_2O$	180
$(NH_4)_6Mo_7O_{24}*4H_2O$	20
$CuSO_4*5H_2O$	80
$FeSO_4*7H_2O$	5000
$CoCl_2$	250
H_3BO_3	1000

Add 1ml of conc. H_2SO_4 per 1L, stir until dissolved and filter-sterilize (*do not autoclave!*).

Kao-Michayluk vitamin mixture (100X) in mg/L.

p-Amino benzoic acid	2
L-ascorbic acid	200
Biotin	10
D-calcium pantothenate	100
Choline chloride	100
Folic acid	40
Myo-Inositol	10000
Nicotinamide	100
Pyridoxine hydrochloride	100
Riboflavin	20
Thiamine hydrochloride	100
Vitamin A (retinol)	1
Vitamin B12	2
Vitamin D (cholecalciferol)	1