Shuttle Vector:

Most important

1. origin of replication for each organism.
2. selectable markers for each organism
3. promoter elements that work in the different organisms

also:

4. screening markers
5. MCS
6. if shuttle vector for eukaryotic: things like enhancers, poly A signal, an intron

Importance of UTRs

1. 5' contains signals for translation: in proks – shine delgarno; in euks – 5' cap (or internal ribosome entry sites), Kozak motifs
2. 3' contains signals for mRNA stability: most notably in eukaryotes with the poly A signal and poly-A tail.
3. UTRs are transcribed, but not translated: they can not contain basal promoter elements (though they can contain enhancer sequences, sometimes) and unless you stated enhancers, they can not have a direct effect on transcription, only on translation.

Protein engineering and evolution

1. evolution works by tailoring organisms and their components to fit into individual niches (temperature, pH, etc…); this is exactly what we are trying to do when performing protein engineering.
2. evolution works by mutations, some of which affect the "performance" of cellular components under different conditions. This is exactly how protein engineering works.
3. we can learn from observing changes to proteins that have occurred in evolutionary time to gain insight on how to modify existing proteins thereby mirroring this evolutionary process (ie. Mother nature has been engineering proteins to fit different niches forever)

Hollow Fibre Bioreactor

1. the most important thing: the semi-permeable membrane to separate the cells and products from the continuous flow of media; allows for waste and CO2 to diffuse out, new nutrients and O2 to enter, cells and products can't escape through the membrane and remain in the growth chamber
2. controlled entry points for media, controlled exit points for cells and product: can harvest without disturbing the rest of the culture
3. can be used to produce Ab and yields concentrations comparable to ascites tumours in mice.

Inclusion Bodies are….

1. evil: IB are aggregates of denatured and mis-folded protein. If your protein aggregates into IB, you will need to solubilize the IB (not hard to do) but because the proteins were denatured in the first place, you then have to attempt to re-fold them. This is not an efficient process at the best of times and sometimes it is not possible at all.
2. good: can be exploited for purification because if you protein ends up in IB, the aggregates can be recovered by centrifugation and you don't have as many contaminating proteins to separate from. Also, toxic proteins can be shunted to IB thereby rendering them harmless before they have a chance to kill the host. If you can refold (or if you don't need perfectly re-folded protein), then IB are peachy.

Growth Kinetics of fermentor and protein production:

This answer had nothing to do with growth conditions. Although growth conditions do affect the growth kinetics, the question was about kinetics alone !!!

1. during lag phase, cells are adjusting to growth conditions, not really producing your product of interest
2. during log phase, cells are actively dividing and turning most of the nutrients into more cells, not producing the product of interest
3. during stationary phase, culture has pretty much reached saturation. Cells are no longer dividing very actively, sustained but moderate feeding at this stage will keep the cells alive but will mean that most of the nutrients will be turned into the product of interest
4. death phase means cells are dying and yield will not be very good

Comparison of protein sequences is useful

1. comparisons can enable you to look for conserved domains or catalytic residues
2. homology can be used to indirectly infer the function of a new protein
3. homology can be used to infer evolutionary relationships
4. protein alignments are more useful than DNA alignments because they align the proteins in the context of a reading frame

How can we do site-directed mutagenesis

1. Anneal a primer(s) to a single stranded plasmid. Primer has mutation in the middle. Extend with polymerase and seal with ligase, transform into E.coli. Due to semi-conservative replication, half of the colonies will contain mutation. If you use a secondary primer (a selection primer – restriction site, mutation of a selectable marker) you can use this selection to select your mutant plasmids
2. PCR-based mutagenesis: design PCR primers with 5' mismatches to the template, perform PCR using mutagenic primers

Protein separation exploits differential physical properties, give examples.

Some of the physical properties that can be used for differential separation: MW, 3-D size and shape, pI, overall charge or surface charge, overall or surface hydrophobicity

- Use MW in SDS-PAGE
- Use pI in IEF
- MW and pI in 2-D gel electrophoresis
- Use surface hydrophobicity with HIC; overall hydrophobicity with RPC
- Use surface charge with IEC, overall charge with native PAGE (which also uses 3-D shape)
- Surface hydrophobicity exploited by salting out
- Size (and shape) is used in size exclusion chromatography

Cell genetics and recombinant protein productioin, give examples

A lot of you mistook this to mean talking about the different expression systems. This isn't really genetics (it is indirectly, which is why many of you got part marks; genetics is about mutants and this question was about using different mutant backgrounds to your advantage in protein production). Nonetheless, if all you described was the different expression systems (E.coli, Caulobacter, Yeast, Pichia, mammalian, BEVS), I gave you decent part marks. Some of you actually did talk about genetic quirks exploited within some of these various expression systems; this got you closer to an ideal answer.

The real expected answer involves some of the mutants we did discuss in class:

- use of cells deficient for HGPRT when making hybridomas
- use of cells that are immortalized (for hybridomas or anything else, really)
- use of cells deficient in protease production
- use of cells that produce less ammonia from glutamine
- use of cells that are deficient in apoptotic pathways
- this answer was clever: using camelid single chain antibodies which are smaller but have same diversity and specificity as regular Ab (these single chains are nothing but mutant versions of full-chain Ab)

Describe the various chromatographic separation techniques, When are they used in industrial contexts ?

- IEC: Cation and anion exchange columns: use of buffers that lead to charge or loss of charge and reduction or enhancement of interaction with the matrix
- HIC and RPC: use hydrophobicity; Surface hydrophobicity used for HIC, use of chaotropes to reduce or enhance binding to matrix; for RPC use non-polar solvents, use total hydrophobicity
- Size exclusion chromatography: uses sieving matrix to separate according to size
- Affinity chromatography: using a ligand interaction to bind the protein.

Many of you forgot to point out how these various techniques can be applied to industrial settings: In most cases, IEC and HIC will be used as intermediate methods to enhance the purity of a crude protein precipitation obtained from an AmSO4 ppt, followed by a final polish/buffer exchange with SEC. Affinity Chr could be used anywhere along the way, probably still followed by SEC. RPC is only used for peptides, not intact protein. The idea, move from high capacity/low resolution methods to low capacity/high resolution methods.

Interaction between X and Y, how do you determine it happens and which protein it is ? set up diagnostic test for it.

There are many ways to go about this. We first need to detect the interaction, once we can detect it, we need to develop tools for a diagnostic test. We can all pretty much agree that the diagnostic test is going to have to be some sort of Ab-based test, which means we will need to first detect the interacting protein, then develop an Ab for it. The best approach would be the one that could most quickly and easily applied to both tasks.

- The preferred approach: use two-hybrid method to detect the interaction. Why ? once you have isolated the interacting protein, you have the sequence to be able to mass produce the protein for subsequent Ab production. To make the Ab, why not pan a phage-display library instead of making Ab? affinity maturation is easier than making and screening hybridomas, by a long shot !!!! And the phage display clones are more than adequate for diagnostics. As for the diagnostic test, the quickest is probably an ELISA, the most accurate would be a western.

Variants on detection of interaction:

- couple protein X to matrix, run serum through affinity matrix, interactor would be retained. Elute and identify by MS or peptide sequencing (this part of subsequent identification makes this method inferior to using two-hybrid). Purify **sufficient** amounts for antibody or phage display selection (this is why this method is inherently inferior to the one above).
- run serum samples on 2-D gel, blot onto membrane, probe with labelled protein X, or probe with unlabelled protein X then detect using labelled anti-X Ab. Once positive spot has been identified, need to ID protein by MS or peptide sequencing. Then need to isolate enough for Ab (or phage) screening.

In the end, if your method was viable and you didn't gloss over parts of your methodology then you would get full marks.

Bind-Wash-Elute: give examples

- Panning a library then performing affinity maturation
- Screening hybridomas for mAb
- Any sort of column-based chromatography (except for size exclusion)
- Although blotting (western, southern, northern) and some immuno-based assays (ELISA, expression library screening with Ab) do not require elution, they do work on the bind/wash principle

Phage display makes great binder, how do you humanize it ?

- must change everything but the CDRs into human antibody sequences
- clone the VL and VH sequences from FAB fragment into a shuttle vector that contains the rest of the antibody sequences (CL, CH1, CH2 and CH3 regions) to produce mostly-human chimeric Ab
- This construct will need to be expressed in a mammalian cell or else the Ab won't fold properly: the best bet, make a transfectoma by transfecting myeloma cells.

Why do mammalian cells suck at recombinant protein production ?

- Many people forgot what I consider to be the **most crucial** part of the answer: the difficulty in creating a stable transfection: integrated DNA into active chromatin. People, this is an extremely low probability event ! Without it, you need take your chances transfecting new cells everytime. This is bad in an industrial setting !!! Couple this with the fact that the best methods for transfecting mammalian cells require expensive reagents (liposomes). This is why even if you gave me 200 other reasons, if you missed this one I could not give you 10/10.

Other factors:

- overall physical wussyness: O2, pH, temperature, shear forces, sensitivity to impurities in water, ammonia and lactate, etc…
- Requirement for rich complex media that can't have antibiotics (potential contamination problems)
- Slow doubling times and low production means continuous cultures (more potential contamination problems)
- Need lots of surface attachment so can't reach very high cell densities
- Apoptosis