TECHNICAL UNIVERSITY OF MOMBASA

FACULTY OF APPLIED AND HEALTH SCIENCES

DEPARTMENT OF PURE & APPLIED SCIENCES

UNIVERSITY EXAMINATION FOR:

MASTERS OF SCIENCE IN BIOTECHNOLOGY

ABT 5109: ADVANCED MICROBIAL DIVERSITY AND SYSTEMATICS

SPECIAL/ SUPPLEMENTARY EXAMINATIONS

SERIES: SEPTEMBER 2018

TIME: 3HOURS

DATE: Sep 2018

Instructions to Candidates

You should have the following for this examination

-Answer Booklet, examination pass and student ID

This paper consists of **SIX**Question(s). Attemptany FOUR questions.

Do not write on the question paper.

Question ONE

a. Explain factors that affect transformation efficiency in bacteria (the factors 15mks)
b. Highlight the major steps in plasmid cloning using restriction enzyme (10mks)

Question TWO

a. Discuss the stages of biofilm forming process in bacteria (10 mks)
b. Explain quorum sensing pathways used by Gram negative bacteria (15mks)

Question THREE

a. Discuss the morphological and physiological features used by bacterial taxonomists (13mks)
b. Describe the construction and the use of phylogenetic tress in taxonomy (12mks)

Question FOUR

Describe survival mechanisms employed by archaebacteria in extreme environments (25mks)

Question FIVE

a. Discuss the reasons cyanobacteria are thought to have played a significant role earlier in evolution (5mks)
b. Discuss computer applications in microbiology (20mks)

Question SIX

Discuss the role of microbes in the following biogeochemical cycles:

i. Nitrogen cycle (15mks)
ii. Sulphur cycle (10mks)

Marking scheme

Question ONE

a. A number of factors may affect the transformation efficiency:

Plasmid size — transformation efficiency declines linearly with increasing plasmid size. Individual cells are capable of taking up DNA molecules, and that the presence of multiple plasmids does not significantly affect the occurrence of successful transformation events. 2mks

Forms of DNA — supercoiled plasmids have a slightly better transformation efficiency than relaxed plasmids - relaxed plasmids are transformed at around 75% efficiency of supercoiled ones. Linear and single-stranded DNA however have much lower transformation efficiency. Single-stranded DNAs are transformed at 10^4 lower efficiency than double-stranded ones. 3mks

Genotype of cells — cloning strains may contain mutations that improve the transformation efficiency of the cells. For example, *E. coli* K12 strains with the *deoR* mutation, originally found to confer an ability of cell to grow in minimum media using inosine as the sole carbon source, have 4-5 times the transformation efficiency of similar strains without. For linear DNA, which is poorly transformed in *E. coli*, *recBC* or *recD* mutation can significantly improve the efficiency of its transformation. 3mks

Growth of cells — *E. coli* cells are more susceptible to be made competent at a particular stage of their growth cycle, possibly when the cell volume is the greatest. When preparing competent cells, cells are therefore harvested at particular optical density (normally around 0.4, a higher value of 0.94-0.95 may also be used but this can be impractical when cell growth is rapid.) 3mks

Methods of transformation — The method of preparation of competent cells, the length of time of heat shock, temperature of heat shock, incubation time after heat shock, growth medium used, and various additives, all can affect the transformation efficiency of the cells. The presence of contaminants as well as ligase in a ligation mixture can reduce the transformation efficiency in electroporation, and inactivation of ligase or chloroform extration of DNA may be necessary for electroporation, alternatively only use a tenth of the ligation mixture. Normal preparation of compentent cells can yield transformation efficiency ranging from 10^6 to 10^8 cfu/µg DNA. Protocols for chemical method however exist for making supercompetent cells that may yield a transformation efficiency of over 1 x 10^9. Electroporation method in general has better transformation efficiency than chemical methods with over 1 x 10^{10} cfu/µg DNA possible, and it allows large plasmids of 200 kb in size to be transformed. 3mks

Damage to DNA - Exposure of DNA to UV radiation in standard preparative agarose gel electrophoresis procedure for as little as 45 seconds can damage the DNA, and this can significantly reduce the transformation efficiency. Adding cytidine or guanosine to the electrophoresis buffer at 1 mM concentration however may protect the DNA from damage. A higher-wavelength UV radiation (365 nm) which cause less damage to DNA should be used if it is necessary for work on the DNA on a UV transilluminator for an extended period of time. This longer wavelength UV produce weaker fluorescence with ethidium bromide intercalated into DNA, therefore shorter wavelength (302 or 312 nm) UV radiations may be used for capturing images but such exposure should be limited to a very short time if the DNA is to be recovered later for ligation and transformation. 3mks

b. major steps in plasmid cloning using restriction enzyme

Set up restriction digests for your donor and recipient plasmids.

Isolate your insert and vector by gel purification:

Run your digested DNA on an agarose gel and conduct a gel purification to isolate the DNA. When running a gel for purification purposes it is important to have nice crisp bands and to have space to cut out the bands. Because of this it is recommend that you use a wide gel comb, run the gel on the slower side, and skip lanes between samples. In addition to a DNA ladder standard, it is also a good idea to run an uncut sample of each plasmid to help with troubleshooting if your digests don't look as you expected.

Once you have cut out and purified your insert and recipient plasmid backbone bands away from the gel via your favorite gel purification method, it is important to determine concentration of recovered DNA.

Ligate your insert into your vector:

Conduct a DNA ligation to fuse your insert to your recipient plasmid.

We recommend around 100ng of total DNA in a standard ligation reaction. You ideally want a recipient plasmid to insert ratio of approximately 1:3. Since the number of base pairs for each varies, it is difficult to calculate this based on DNA concentration alone. One method is to conduct 2 ligations for each plasmid you are trying to create, with varying ratios of recipient plasmid to insert.

It is also important to set up negative controls in parallel. For instance, a ligation of the recipient plasmid DNA without any insert will tell you how much background you have of uncut or self-ligating recipient plasmid backbone.

Transformation:

Transform your ligation reaction into your bacterial strain of choice. Follow the manufacturer's instructions for your competent cells.

For most standard cloning, you can transform 1-2µl of your ligation reaction into competent cells such as DH5alpha or TOP10. If using much less total DNA (<1ng) or if you are having trouble getting colonies, you might want to use higher competency cells. Additionally, if your final product is going to be very large (>10kb) you might want to use electro-competent cells instead of the more common chemically-competent cells.

The number of bacterial colonies resulting from your transformation will give you the first indication as to whether your transformation worked. Your recipient plasmid + insert plate should have significantly more colonies than the recipient plasmid alone plate. The recipient plasmid alone control will tell you your "background" level or more specifically it will tell you how many colonies you can expect on your recipient plasmid + insert plate that are not correct.

If you have a high number of colonies on your recipient plasmid alone plate, you can try ligating the recipient plasmid alone in the presence and absence of ligase. If the colonies are a result of uncut empty plasmid, you will still have colonies when you do not add ligase. If the colonies are a result of recipient plasmid self-ligation, you will see significantly more colonies when you add ligase.

If you do not see any colonies, you should conduct a positive control to ensure that your transformation worked. You should also verify that you are plating on the appropriate antibiotic and try varying the amount of recipient plasmid to insert in the ligation reaction.

Isolate the Finished Plasmid:

Finally, you will need to pick individual bacterial colonies and check them for successful ligations. Pick 3-10 colonies depending on the number of background colonies on your control plate (the more background, the more colonies you will need to pick) and grow overnight cultures for DNA purification.

After purifying the DNA, conduct a diagnostic restriction digest of 100-300ng of your purified DNA with the enzymes you used for the cloning. Run your digest on an agarose gel. You should see two bands, one the size of your vector and one the size of your new insert. If you used only one enzyme or used enzymes with compatible overhangs you will need to verify the orientation of your insert, so you may want to design a diagnostic digest for this purpose.

Question TWO

a. Biofilm forming stages: **Biofilm forming process**

The establishment and maintenance of a biofilm community is socially organized and a highly complex process. Formation of a biofilm requires Extracellular polysaccharides (EPS), Lipopolysaccharides (LPS),

both flagellum-dependent and flagellum-independent cell motility, secreted proteins and extracellular DNA.

i) Cell adsorption/ reversible attachment

For a bacteria to attach onto a surface, the surface must be conditioned by possession of some organic substrates e.g. a variety of proteins, polysaccharides and glycoproteins. This conditioning is controlled by surface tension, roughness, wettability and electrophoretic mobility of the surface. Following this conditioning, freely moving bacteria reversibly attach on the surface through microbial adhesion. Only weak forces of attraction exist including Van der Waals, microbial surface charges and other electrostatic forces for interacting molecules hence bacteria can still move away from the surface.

ii) Irreversible attachment

After the attachment of bacteria onto a surface for some time, the nature of the attachment changes from one that is reversible to a non-reversible one. This is through microbial production of cementing substances like EPS which anchor them through a network of fibers onto the surface. The network of fibers become more complex with the inclusion of diversified microbial consortia making the cells stick onto the surface. The complex EPS network provide nutrients from the medium, give necessary cohesive forces for stability of the biofilm while at the same time providing protection from unfavorable environmental conditions.

iii) Maturation/dispersion

A mature biofilm is made up of an elastic part and a viscous part. The elastic part is made up of a matrix structure while the viscous part is made up of water filled interstitial channels and voids which separate microcolonies. The EPS matrix provides the biofilm with mechanical stability through its viscoelastic properties while the viscous part supplies nutrients and oxygen to cells within the biofilm. A variety of major classes of macromolecules like polysaccharides, proteins, nucleic acids, peptidoglycan, and lipids are normally present in a biofilm with extracellular polysaccharides being the major structural components of the biofilm matrix and extracellular DNA helping in the maturation of the biofilm under the control of nucleases.

Bacterial exopolysaccharides exist in either ordered or disordered forms depending on the environment in which they are found. Disordered forms occur where temperatures are elevated and ionic concentrations very low. Polysaccharide molecules can interact with one another or with heterologous ions and molecules to yield gels, a process that require multivalent cations. Polysaccharides also interact with protein molecules both as solutes or when attached to the surface of the microbial cells. The polysaccharide – protein interactions confer structural and functional properties of the matrix as some of the proteins are enzymes constituting an external digestion system. Upon maturity, bacteria use quorum sensing to release single cells into the liquid medium, enabling the biofilm to spread over the surface.

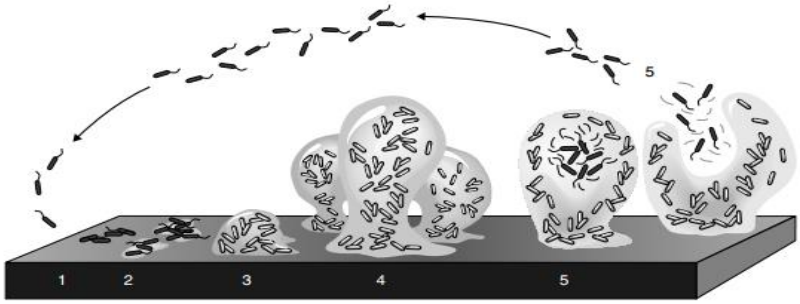
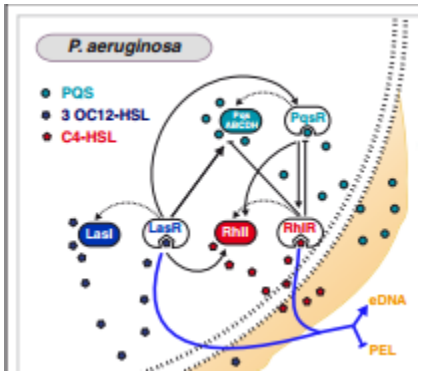


Figure 1.4 Stages of biofilm development: There are five stages of biofilm development. 1. Free swimming bacteria cells attach reversibly onto a surface. 2. If the surface is appropriate, the attachment becomes irreversible through secretion of exopolymeric substances. The cells also lose their motility. 3. Initial formation of biofilm structure. 4. Formation of a complex fully mature biofilm. 5. Release of motile planktonic cells from the biofilm. Adapted from

b. **Quorum sensing pathways in P. aeruginosa/Gram negative bacteria**

As regards QS, P. aeruginosa harbors two complete AHL circuits, LasI/LasR and RhlI/RhlR, being the LasI/R circuit hierarchically positioned upstream the RhlI/R circuit. These two QS systems are composed of a LuxI type synthase, responsible of AHL synthesis, and a LuxR type receptor. At high cell density (HCD), AHLs accumulate and specifically interact with LuxR type transcription factors. AHL binding stabilizes the LuxR type proteins, allowing them to fold, bind DNA, and regulate transcription of target genes. In many cases, AHL bound LuxR type proteins also activate transcription of luxI, providing a signal amplification mechanism via a feed forward autoinduction loop. In addition, P. aeruginosa has two orphan LuxR homologues, VqsR and QscR, and it also presents the Pseudomonas quinolone signal

(PQS), which are interconnected with the LasI/LasR and RhlI/RhlR signaling circuitries.



Question THREE

a. **Morphological and physiological features used in taxonomy**

• Colony morphology**(1),** Colony color**(1)**, Cell shape**(1),** Staining results (Gram) **(1),** Endospore**(1)**, Motility**(1),** Flagella**(1),** Size**(1),** Cellular inclusions**(1)**, Ultrastructure**(1),** Oxygen relationship**(1),** Energy sources**(1),** Electron sources**(1),** Carbon sources**(1)**, Fermentation products**(1),** Cell wall components**(1),** Temp and pH optima**(1),** Osmotic tolerance**(1),** Photosynthetic mechanism**(1),** Secondary metabolites**(1)**, Sensitivity to antibiotics**(1)**

b. **Phylogenetic tree and its construction**: A phylogenetic tree, also known as a phylogeny, is a diagram that depicts the lines of evolutionary descent of different species, organisms, or genes from a common ancestor. Phylogenies are useful for organizing knowledge of biological diversity, for structuring classifications, and for providing insight into events that occurred during evolution. Furthermore, because these trees show descent from a common ancestor, and because much of the strongest evidence for evolution comes in the form of common ancestry Allows present-day researchers to use phylogenies to visualize evolution, organize their knowledge of biodiversity, and structure.
Trees provide an efficient structure for organizing knowledge of biodiversity and allow one to develop an accurate, nonprogressive conception of the totality of evolutionary history.

Question FOUR

- Heat stable proteins that have more hydrophobic interiors; prevents unfolding or denaturation at higher temperatures; Chaperonin proteins; maintain folding; Monolayer membranes of dibiphytanyl tetraethers; saturated fatty acids which confer rigidity, prevent degradation in high temperatures; A variety of DNA-preserving substances that reduce mutations and damage to nucleic acids; e.g., reverse DNA gyrase and Sac7d; Can live without sunlight or organic carbon as food; survive on sulfur, hydrogen, and other materials that other organisms cannot metabolize; Proteins rich in α-helices and polar groups; allow for greater flexibility; “Antifreeze proteins”; maintain liquid intracellular

conditions by lowering freezing points of other biomolecules; Membranes that are more fluid; contain unsaturated *cis*-fatty acids which help to prevent freezing; active transport at lower temperatures; Survive high salt concentrations by; interacting more strongly with water such as using more negatively charged amino acids in key structures; making many small proteins inside the cell, and these, then, compete for the water; accumulating high levels of salt in the cell in order to outweigh the salt outside; *Deinococcus radiodurans* has from four to ten DNA molecules compared to only one for most other bacteria; Contain many DNA repair enzymes, such as RecA, which matches the shattered pieces of DNA and splices them back together.

Question FIVE

a. Importance of cyanobacteria in evolution: Cyanobacteria have an elaborate and highly organized system of internal membranes which function in photosynthesis**(1)**. Cyanobacteria get their name from the bluish pigment phycocyanin**(1)**, which they use to capture light for photosynthesis. Photosynthesis in cyanobacteria generally uses water as an electron donor and produces oxygen as a by-product, though some may also use hydrogen sulfide as occurs among other photosynthetic bacteria. Carbon dioxide is reduced to form carbohydrates via the Calvin cycle. In most forms the photosynthetic machinery is embedded into folds of the cell membrane, called thylakoids**(1)**. The large amounts of oxygen in the atmosphere are considered to have been first created by the activities of ancient cyanobacteria. Due to their ability to fix nitrogen in aerobic conditions they are often found as symbionts**(1)** with a number of other groups of organisms such as fungi (lichens), corals, pteridophytes (Azolla), angiosperms.

b. Computer applications in microbiology: **Computer Applications in Microbiology:**
Computers can serve a variety of functions in fermentation process control and analysis.

i. Optimisation via Computer:

Computers are used in scale up, to store and evaluate fermentation parameters and to measure the effects of individual parameters on the metabolic behaviour of cultures.

ii. Control via Computer:

Computers can control fermentation processes. On-line fermentation control is widely used in the production scale in many companies.

Computer applications in microbiology not yet as widespread as in the chemical industry for several reasons. Sensors suitable for use in sterile systems are not yet reliable enough to take advantage of computer capacity and biosynthesis.

Regulation of metabolite formation is not yet fully understood. The fermentation cost reduction by using computers is difficult to calculate. Thus, in microbiology, computers are used primarily for data acquisition, data analysis and development of fermentation models.

(a) Data acquisition:

Data can be acquired directly at the fermenter with on line-sensors. The information's acquired can be such as pH, temperature, pressure, viscosity, fermenter weight, power uptake, aeration rate and O_2 and CO_2 content in the gas stream. Other data can be obtained from laboratory measurements and fed into the computer off-line, e.g. biomass concentration nutrient content, metabolite formation.

This information can be entered as raw data and can be converted by the computer to standard units, for example to adjust volumes for a standard temperature, temperature correction data can be used to calculate the true aeration rate for a production system.
An alarm system can be looked up to the data-acquisition system to inform the attendant when deviations from standard value occur. Data about the course of fermentation can be stored, retrieved and printed out and product calculations can be documented.

(b) Data Analysis:

The data entered or measured is used in calculations such as CO_2 formation rate, O_2 uptake rate, respiratory quotient, specific substrate uptake rate, yield coefficient, heat balance, productivity, volume-specific energy uptake, and Reynold's number.
When biomass is not continuously measured, the biomass concentration can be calculated through the O_2 uptake rate. It is assumed that the yield constant and the proportion of O_2 needed for maintenance metabolism are known. The calculations must be adjusted if, for example secondary metabolites are formed or the yield constant changes during the fermentation.
After fermentation at different pH, and temperature levels, "isoproduction and isotime curves" are computed. The fields are given as percentage of the minor production compared with the max-erythromycin titre. Optimal productivity (production/fermentation time) for a given set of operating conditions can be ascertained by this graph.

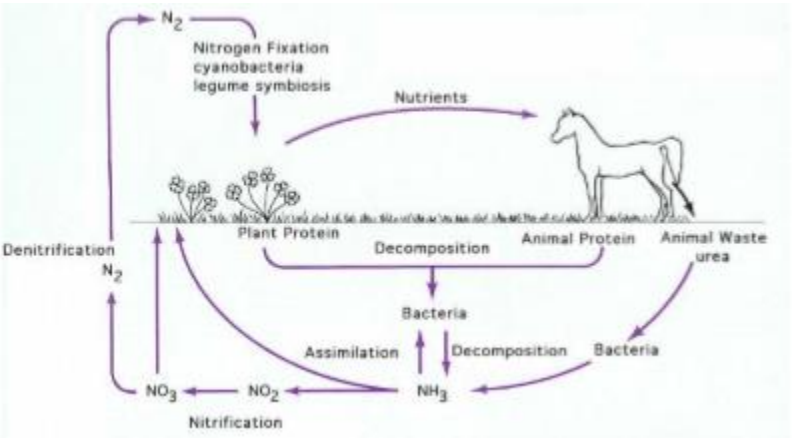
iii. Software:

A good number of software's are available in the market such as MENTOR by LSLBILAFITTE, MFCSAVin by B. BRAUN BIOTECH, AFS BioComond by New BRUNSWICK SCIENTIFIC which are used in laboratory as well as pilot or industrial scale fermenters. Most of the softwares are supplied by manufacturers of fermenters. Some of the software's are able to detect automatically the configuration of controller in fermenters.
These software's are also used in regulating the dilution rate, calculating the growth rate, interfacing with other devices such as analyses and archiving of data for validation. These software programme loggings are generally written in Basic, Pascal or C languages. But to overcome various difficulties, MS window is being used as a general platform for manufacturing of software programmes for laboratory and NT version for large scale fermenters.
The significance of such devices helps to secure interface between the external programmes based in MS window and real time programme. Several different control approaches have been developed for model-based control strategies and adaptive control; thus it offers more scope for effective control of fermentation processes.

Question SIX

i. Nitrogen cycle: N2 has 3 bonds making it very stable and energy is required to break the bonds. The nitrogen in the air becomes a part of biological matter mostly through the actions of bacteria and algae in a process known as nitrogen fixation. Legume plants such as clover, alfalfa, and soybeans form nodules on the roots where nitrogen fixing bacteria take nitrogen from the air and convert it into ammonia, NH3. The ammonia is further converted by other bacteria first into nitrite ions, NO2-, and then into nitrate ions, NO3-. Plants utilize the nitrate ions as a nutrient or fertilizer for growth. Nitrogen is incorporate in many amino acids which are further reacted to make proteins.

Ammonia is also made through a synthetic process called the Haber Process. Nitrogen and hydrogen are reacted under great pressure and temperature in the presence of a catalyst to make ammonia. Ammonia may be directly applied to farm fields as fertilizer. Ammonia may be further processed with oxygen to make nitric acid. The reaction of ammonia and nitric acid produces ammonium nitrate which may then be used as a fertilizer. Animal wastes when decomposed also return to the earth as nitrates. To complete the cycle other bacteria in the soil carry out a process known as denitrification which converts nitrates back to nitrogen gas. A side product of this reaction is the production of a gas known as nitrous oxide, N2O. Nitrous oxide, also known as "laughing gas" - mild anesthetic, is also a greenhouse gas which contributes to global warming.



Nitrogen fixing bacteria include

Azotobacter, some Bacillus, Klebsiella, Rhodospirllum (photosynthetic) and Anabaena, Nostoc (cyanobacteria, blue-green algae) fix N2 directly- free-living N2 fixers

Rhizobium, Spirillum, Frankia alni are symbiotic N2 fixers with specific hosts viz roots of leguminous plants, tropical grasses and alder trees

Symbiotic and free-living fixation requires nitrogenase enzyme and the following reaction occurs:

Nitrogen + nitrogenase ------> 2 molecules of ammonia ------> incorporation into amino acid

Azotobacter - heterotrophs; more carbon means more fixation

Rhodospirillum - phototroph; light dependent fixation

Rhizobium - host specific fixation

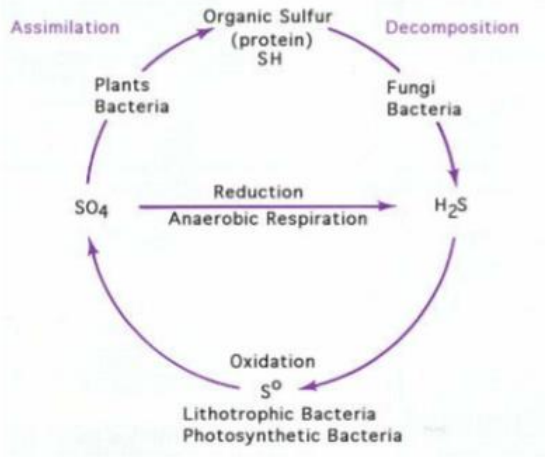
Nitrogen fixation occurs under anaerobic conditions only & nitrogenase enzyme has to be protected from oxygen:

leghemoglobin (synthesised jointly by Rhizobium and the plant) - binds 02

heterocysts - protective compartment in cyanobacteria

cysts (membranous structure) - Azotobacter
Fixation only if anaerobic conditions present - Klebsiella
Two approaches to increase N2 fixation:
Genetic approach: Introduce nitrogen fixing genes (nif genes) into non- N2 fixing plants (eg corn, wheat which provide good carbohydrates); Problems include protecting nitrogenase from 02 and energy required for fixation will be used from the plant at the expense of plant growth.
Microbial ecology: Isolation of new strains of N2 fixers (increase biodiversity)and establish new symbiotic relationships or new associations between plants and N2 fixing bacteria.
(b) Ammonification:
Fixed nitrogen that is locked up in the protoplasm (organic nitrogen) of N2 fixing microbes has to be released for other cells. This is done by the process of ammonification with the assistance of deaminating enzymes.
Alanine + deaminating enzyme --------> ammonia + pyruvic acid
It is ammonia rather than the organic nitrogen that is required by most plants and hence the role of microbes in the process is extremely important.
(c) Nitrification:
The most widely used inorganic nitrogen source for plant and animal growth is nitrate.
The production of inorganic nitrate is a 2 stage process:
NH4+ ------> NO2 (partial oxidation of NH4+): eg Nitrosomonas; chemolithotrophs obtain energy by aerobic oxidation of inorganic compounds and cell carbon by CO2 fixation
NO2 ------> NO3 eg Nitrobacter
(d) Denitrification:
NO3 is a +5 oxidation state ie highest state possible and returns to 0 (zero) state by denitrification process which is a sequential addition of electrons to NO3 to produce N2.

ii. Sulphur cycle



There are 4 chemical forms of sulfur: Elemental sulfur (So): oxidation state of 0
Sulfide (H2S, FeS): oxidation state of -2
Organic mercaptans, aminoacids (meth, cyst): oxidation state of -2

Sulfate (H2S04): oxidation state of +6
All transformations are microbially mediated
All transformations depend on the oxygen content in the environment So in aerated environments ----------> S04 So in anaerobic environments -------> H2S
Begin looking at the sulfur cycle from the organic sulfur (eg meth, cyst, certain vitamins) from dead organisms which decompose by microbial enzymes in the release of H2S.
The H2S release is either:
released into the environment OR
reacts with metals to form FeS (ferrous sulfide)
H2S and FeS are in their most reduced oxidation state and are oxidised to SO4; Two different metabolic pathways operate in microbes for oxidation Two stepped process in which H2S and FeS are oxidised to elemental So first (intracellular eg phototrophic Chromatium; or extracellular eg Ectothiorhodospira, Chlorobium) and than to SO4
In Thiobacillus, H2S and FeS are oxidised to cellular sulfhydryl organic compounds, than to sulfides and finally to SO4
The SO4 that is produced reacts with water to form H2SO4. Thiobacillus thiooxidans produces huge amounts of sulfuric acid; an acidophile (optimum pH for growth = 2.0 to 3.5). Advantage: Alkaline soils can be turned into acidic soils by addition of T. thiooxidans and So
Disadvantage: Causes pollution eg pyrite oxidation in coal mines
Finally, SO4 can be used as an electron acceptor and reduced to H2S by sulfate reducing bacteria (SRB), a physiologically cohesive anaerobic microbial group. The group is involved in biocorrosion of metals, the H2S produced is extremely toxic ad is known to kill total fish populations of organically rich (polluted) lakes

COURSE OUTLINE

ABT 5109: Advanced Microbial Diversity and Systematics

Microbial systematics: classical versus modern classification. Overview of microbial diversity (Bacteria Eukaryota Viruses). Archaea: Archaeal diversity, unique molecular and biochemical features, phylogenetic overview of Archaea. Euryarchaeota, Crenarchaeota and Nanoarchaeota. Extremophiles; biology, habitat and economic importance of the various groups. Ecophysiology and ecological niches of microbes. Structure and functions in microbial communities. Microbial biofilms. Habitats of prokaryotes: aquatic, sediments, soils, extreme environments. Microbial associations with animals. Microbes and plants. Microbial biogeochemical cycles. Methods in microbial diversity analysis. Ecological genomics. Recent trends in microbial taxonomy: Computerized identification of bacteria, chemotaxonomy, genetic methods in taxonomy- PCR and DNA fingerprinting, DNA base composition and hybridization.