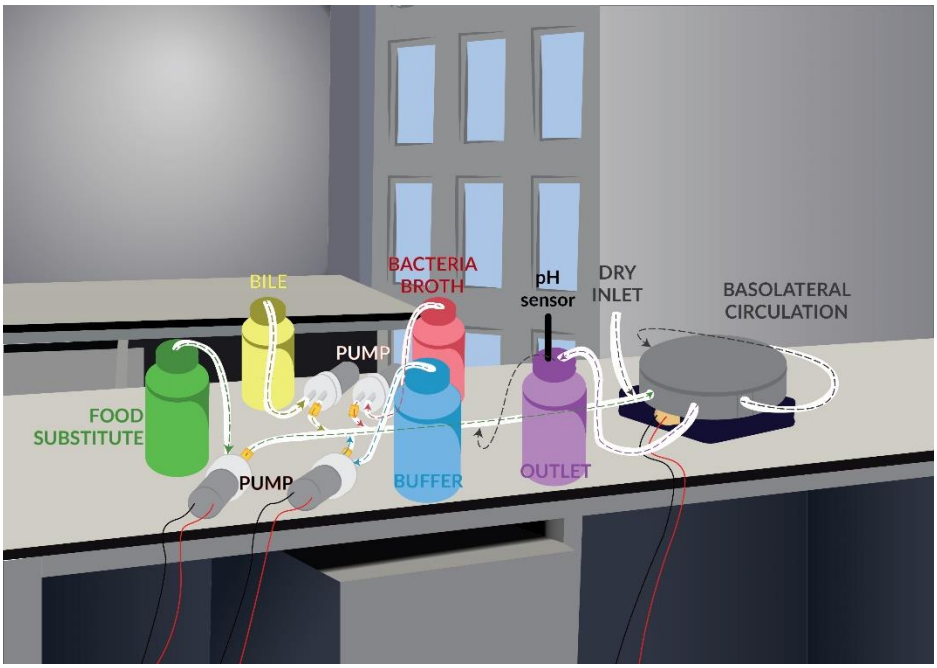
iGEM MIT_MAHE
SAUL Handbook

Acknowledgements

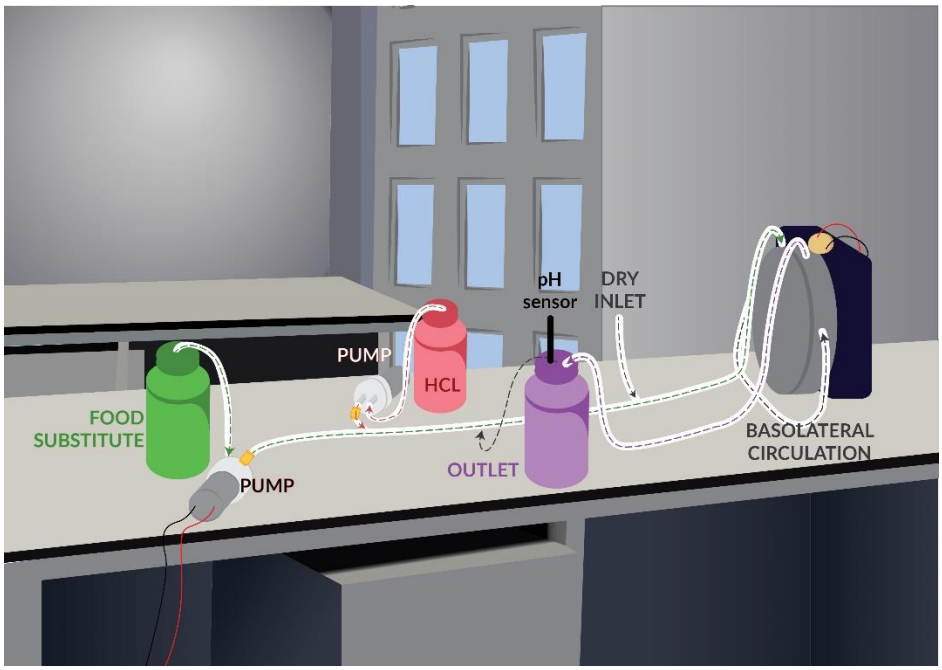
The curation of this handbook by Urvi Gupta and Beejady Adithi Somayaji of iGEM MIT_MAHE would not have been possible without the exceptional advice of our Dr. Keyur Raval. His enthusiasm and knowledge kept the information and design relavent to the use. Nishesh Singh, Parthivi Choubey and Sajan Subramaniam, our fellow colleagues helped in various aspects like design, electrical requirements and illustrations. Soumodeep Sarkar, our fellow teammate, has also given us insight into the experiments and answered with unfailing patience numerous questions about the content. We thank Urvi Gupta for designing, design parameters, in silico analysis, safety, writing and editing, Beejady Adithi Somayaji for writing, editing, experimentation, design parameters and safety. The expertise and skills of everyone involved has improved this handbook in innumerable ways.

Table of contents

1. Working, parameters and model design



Horizontal configuration (intestine)



Vertical configuration (stomach)

A. Introduction:

The small intestine is a complex dynamic environment with a variety of physiological and mechanical stresses in play. These properties play an important role in determining the viability of the probiotic drug components. In vivo studies are too complicated and expensive to be used for the initial screening processes like studying the chassis to be used, checking the effectiveness of the probiotic in a variable dynamic environment and/or in optimization of the capsule for appropriate delivery. Current lab scale methodologies involve a relatively oversimplified static system which would not replicate most properties of its target site environment. They do not subject the organisms to continuously varying stresses which the organisms experience in vivo. **Systematic Analyzer of Underlying Limitations (SAUL)** aims to provide more variable conditions to combat as well as study the underlying limitations of current static methodologies.

Components of GI tract:

Physiological conditions and anatomy of the organs (stomach and intestine) including length, amount of enzymes released, pH, flow rates etc. varies from person to person. This model takes into account relevant averages and approximations.

Stomach:

Stomach is a hollow organ - the part of the gastrointestinal tract involved in the digestion and sterilization of food substances. It secretes a variety of gastric juices and HCl, and has a low pH range of 1.5 to 3.5.

There are three types of movements involved:

- Peristaltic wave
- Segmentation
- Ancillary movements

In this model we would be focusing on only two types of movement i.e Peristalsis and Ancillary. The pH would be maintained at 2.

Note: The pipe dimensions would not be in scale to the stomach geometry. Hence the flow pattern would differ in the stomach.

Peristalsis: Peristalsis is the radially symmetrical contraction and relaxation of the muscles of the gastrointestinal tract in a unidirectional wave-like motion.

Ancillary movement: It is the state of complete relaxation of the stomach to allow accumulation of the food/bolus.

Small intestine:

Duodenum is the first part of the small intestine located between the stomach and the jejunum. One of the most important physiological characteristics of the intestine is pH which is basic/close to neutral because the lining of the small intestine is more delicate as it is involved in absorption. It is the first site of action of our probiotic where our capsule would dissolve, and the probiotic would be released into the gut environment.

Like stomach, the main types of muscle movement contributing to intestine motility are peristalsis and segmentation.

Note: Segmentation will not be modeled in SAUL.

In duodenum there is a sudden change in pH to 6. Here there would be the presence of bile juices contributing to the viability of the probiotic.

Jejunum and ileum are the parts of the intestine solely involved in absorption. They are located between the duodenum and the large intestine.

In jejunum the pH further increases to 7-9 in the jejunum and averages to around 7.4 in the ileum.

Materials used:

For reactor:

Body	ABS (Acrylonitrile Butadiene Styrene) filament	Link
Motor	DC motor with worm gearbox	Link
Tube (Inside the reactor)	Dialysis tube	Link
Tube (Outside)	Silicone tube	Link
Ball Bearings (3)	Ball bearings	Link
Pump	Pump	Link
Valve	Valve	Link
Chemical container	Containers	Link
M5 Hex Nuts (3)	Nuts	Link
M5 Hex Socket Bolts (3)	Bolts	Link
M3 Hex Socket Bolts (7)	Bolts	Link
M6 rods (4)	Rods	Link

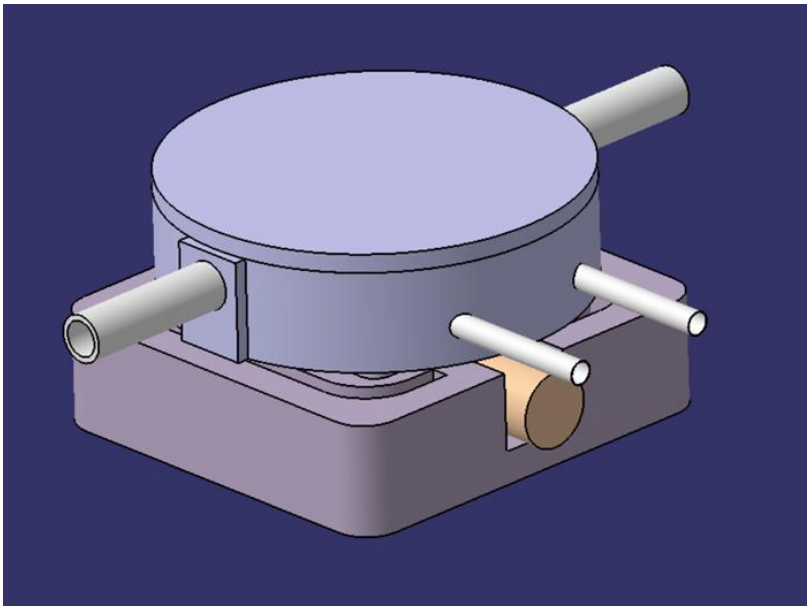
For analysis (Main components):

Food component substitute	Guar gum (0.1%, 0.25%, 0.4%, 0.5%, w/v) or Carboxymethyl cellulose (CMC) (0.1 and 0.5%, w/v), distilled water, NaCl, Luria-Bertani Nutrient broth.
Stomach	0.01M HCl, 1M HCl
pH buffers	$NaHCO_3$
Duodenum (Enzyme)	4% Bile salts
Drug distribution dye	Powdered Indigo dye (Insoluble in water)
Mixing	Magnetic stirrer

Parameters of the organs and food components in consideration:

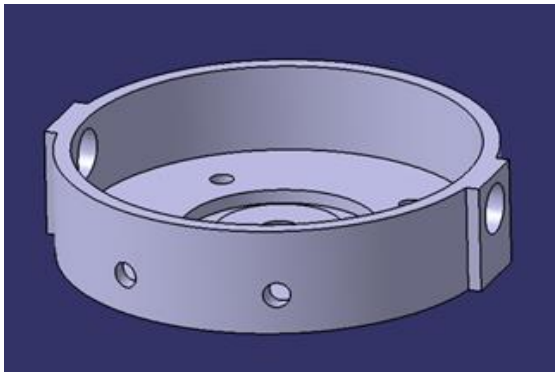
Density	Density of food substitute will depend on the amount of guar gum added.	Link
Viscosity	0.1 - 3.0 Pa.s	Link
Temperature	37°C	Link
pH range	1.5-3.5(Stomach), 6-9(intestine)	Link
Frequency of the peristalsis	3 per minute in the stomach, then 12 per minute in the duodenum, 9 per minute in the ileum	Link
Flow rate	2.5 mL/min to 20 mL/min (Intestine)	Link
Shear stresses	200 Pa	Li, J. et al., (2019)
Capsule diameters	5.31mm and 4.91mm	Link
Retention time	4 hours in stomach, 6 hours in small intestine	Link

B. Design and assembly:

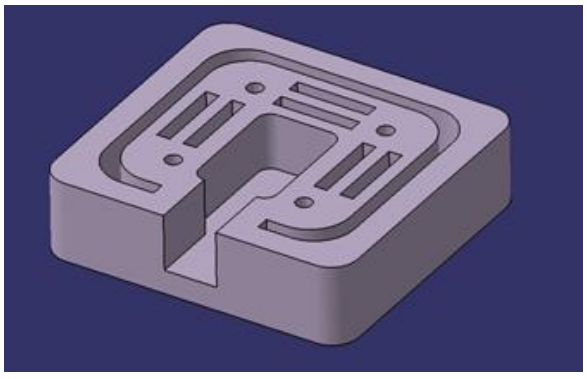


A semi permeable dialysis tube (made of regenerated cellulose) models the apical chamber of the intestine. Imitation of peristalsis is achieved through periodic compression of the dialysis tube by three deep-groove ball bearings. Bearings are used to minimize friction and resulting wear of the tube. The rollers are actuated using a DC motor with a worm gearbox, to satisfy the low rpm requirements. The dialysis tube is fitted into a 3D printed case, which simulates the basolateral chamber. The bottom part of the case holds the motor in place and acts as a stand when the bioreactor is in a vertical configuration. Silicon tubes are used as channels for the basolateral fluids.

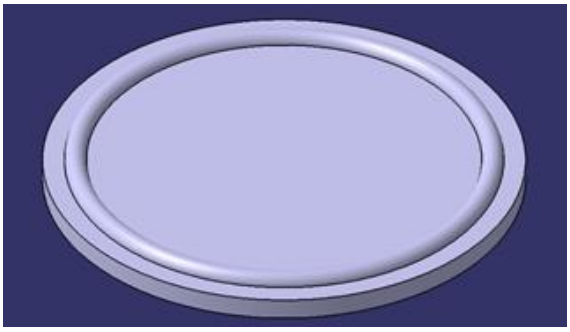
COMPONENTS



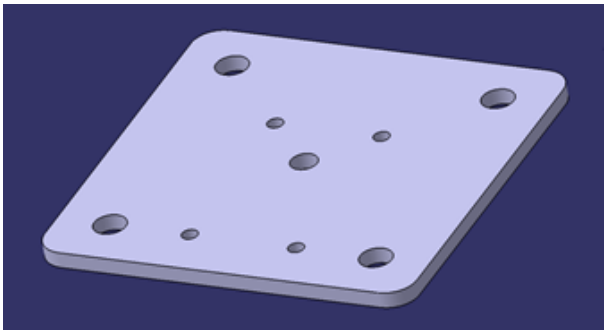
Case top



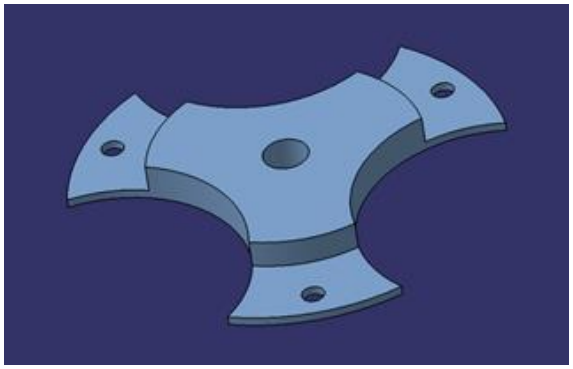
Case bottom



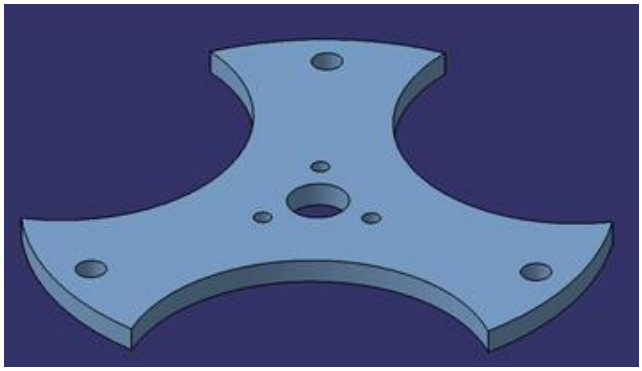
Lid



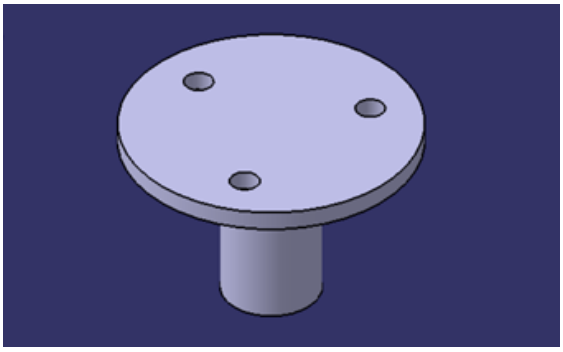
Motor mount plate

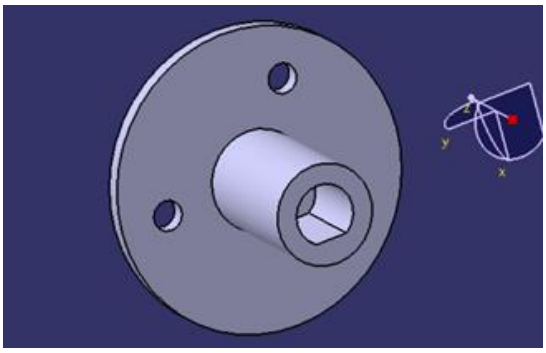


Middle mount

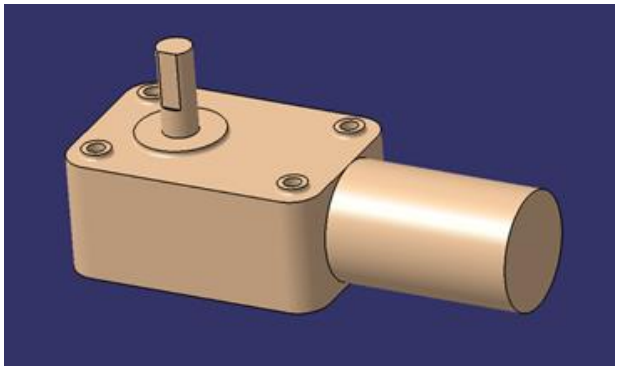


Top plate

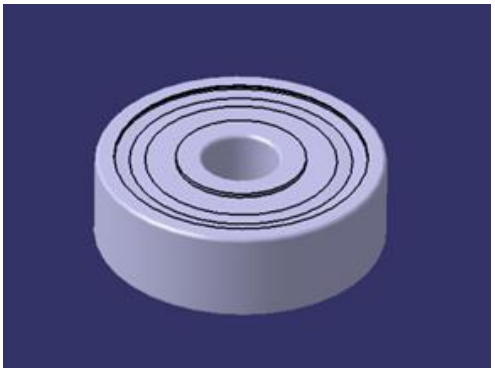




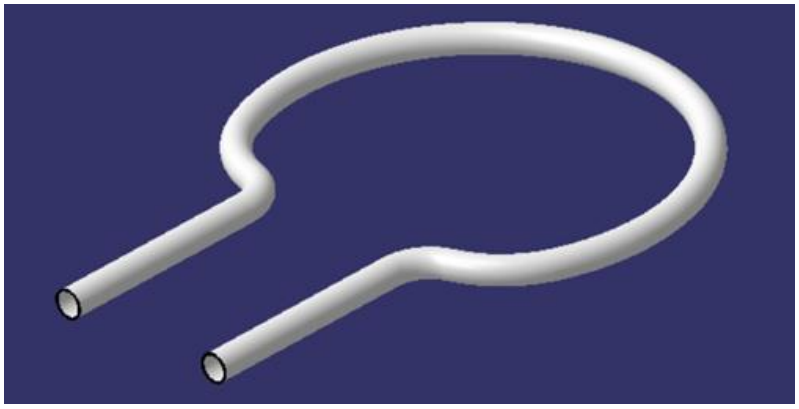
Torque transmitter



Motor

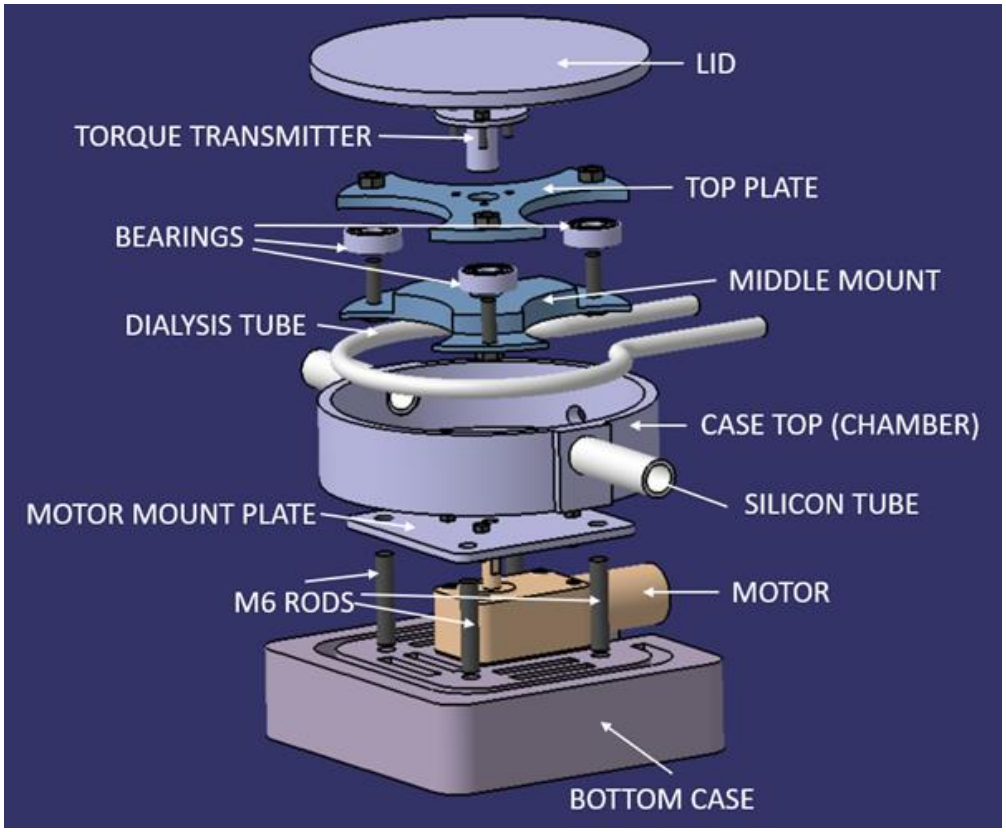


Bearing



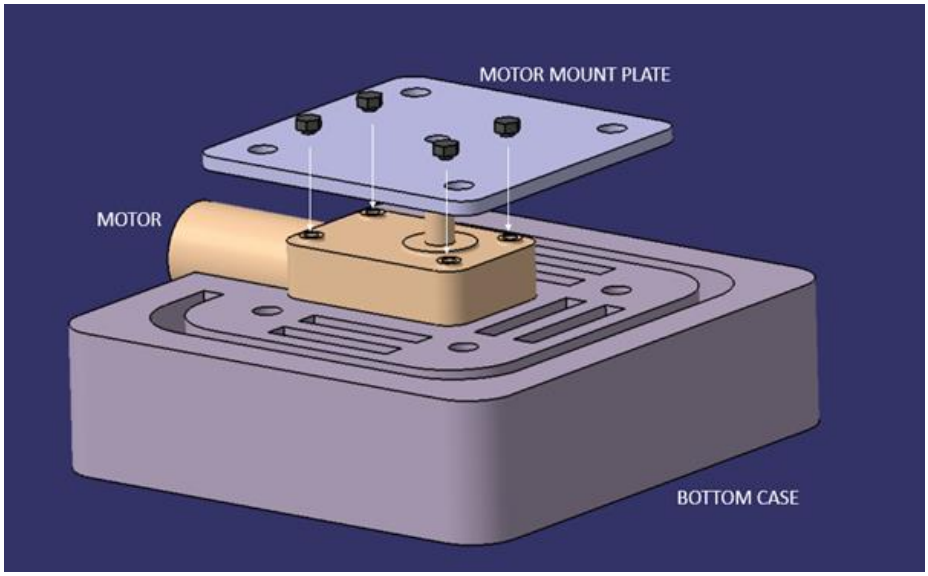
Dialysis tube

ASSEMBLY:

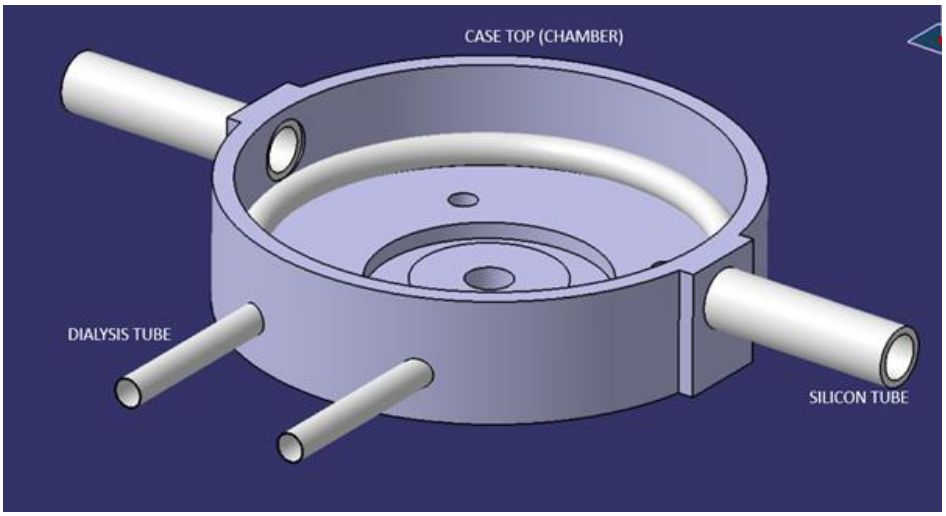


STEPS:

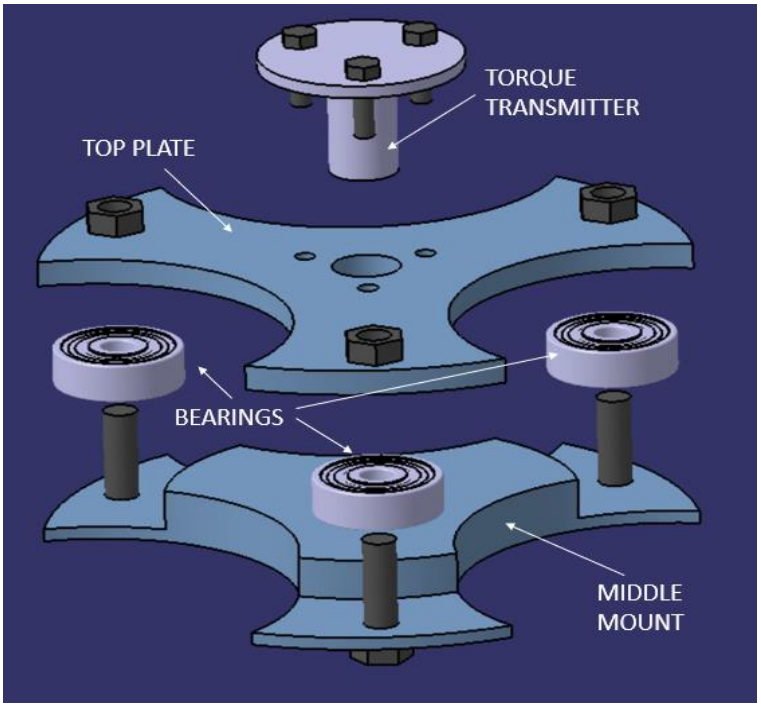
- Motor is bolted onto the motor mount plate, and the plate is secured to the case bottom using the M6 rods.



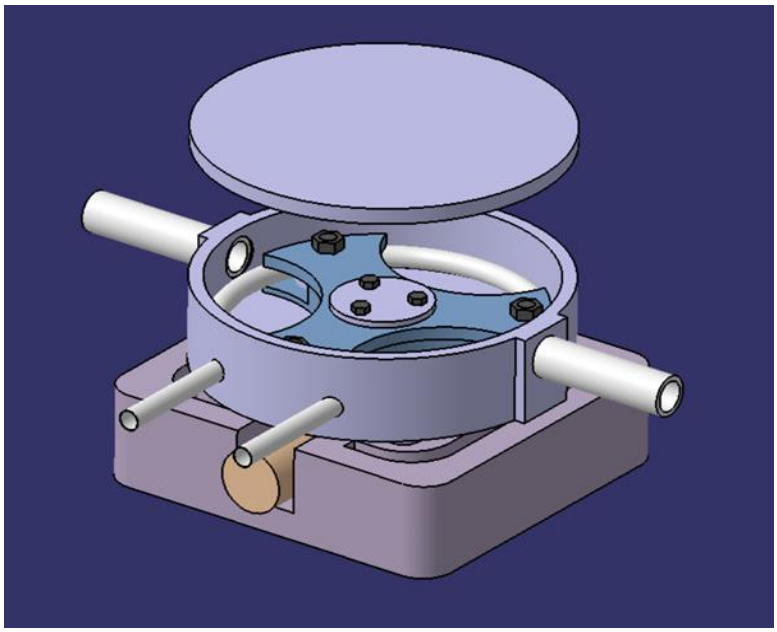
- The dialysis tube is fitted into the chamber.



- The middle mount, bearings, top plate and torque transmitter are assembled separately by placing the bearings on the middle mount, aligning them with the top plate and securing them with M5 bolts. The torque transmitter is fixed to the top plate using M3 screws.



- This assembly is then lowered into the chamber, with the shaft end of the torque transmitter sliding through the hole in the case.
- The chamber, now assembled with rollers and tube, is coupled to the D-shaft motor by lowering it onto the shaft. The D-shaft mates with a corresponding pocket in the torque transmitter.
- The M6 rods provide further support and stabilize the bottom and top parts of the assembly.
- Finally, the lid is attached to seal the chamber.



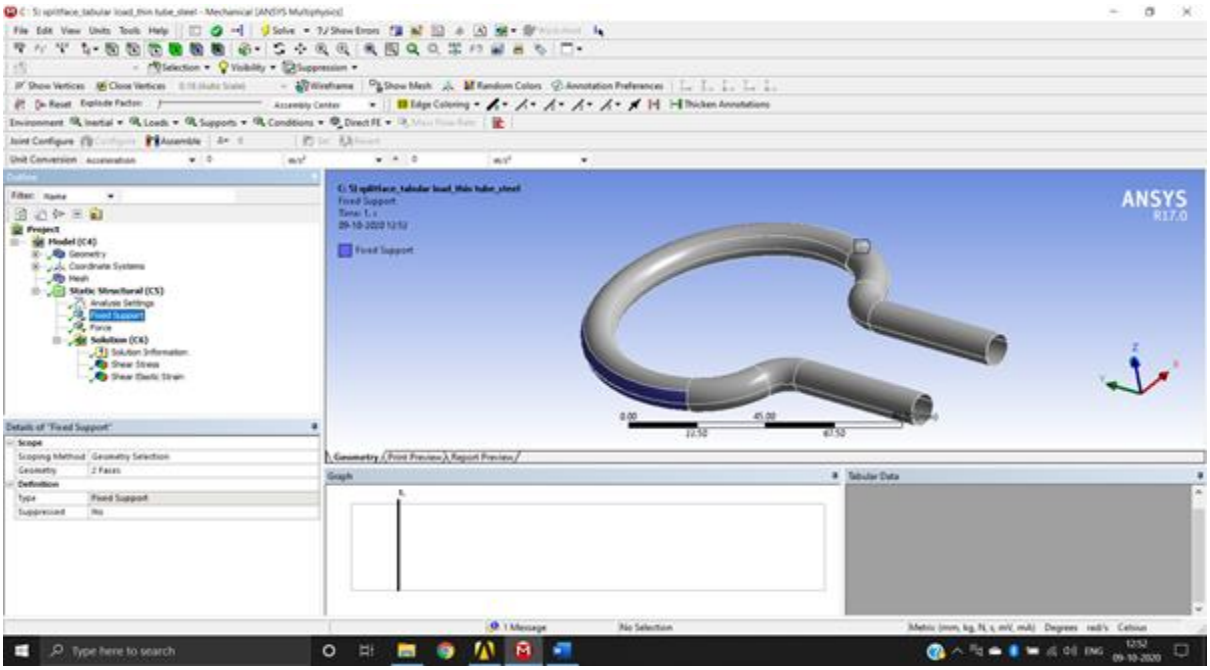
C. In silico Analysis:

Preliminary analysis to check if shear stress is affected by RPM of the motor – Two tests are carried out on a tube of the same dimensions, with same magnitude of compressive force applied to the tube to simulate the roller force, at two different RPM conditions. The RPM is varied by changing the length of the time interval specified for the tabulated load.

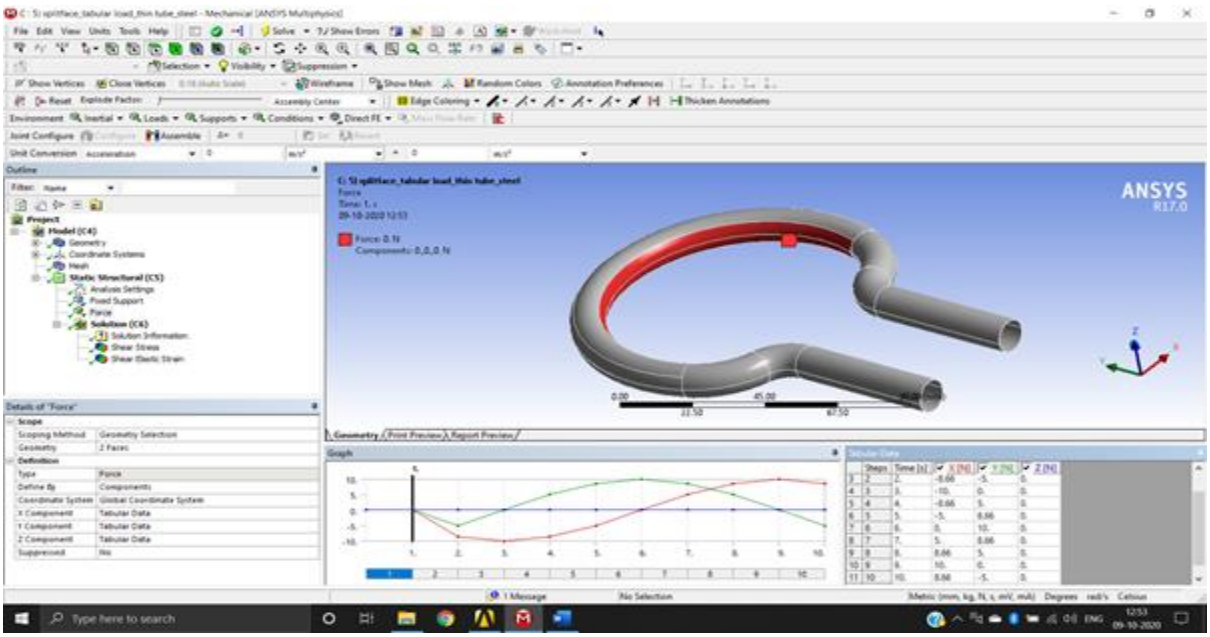
CASE 1: Tube Dimensions OD=11.4mm ID=11mm

Boundary conditions

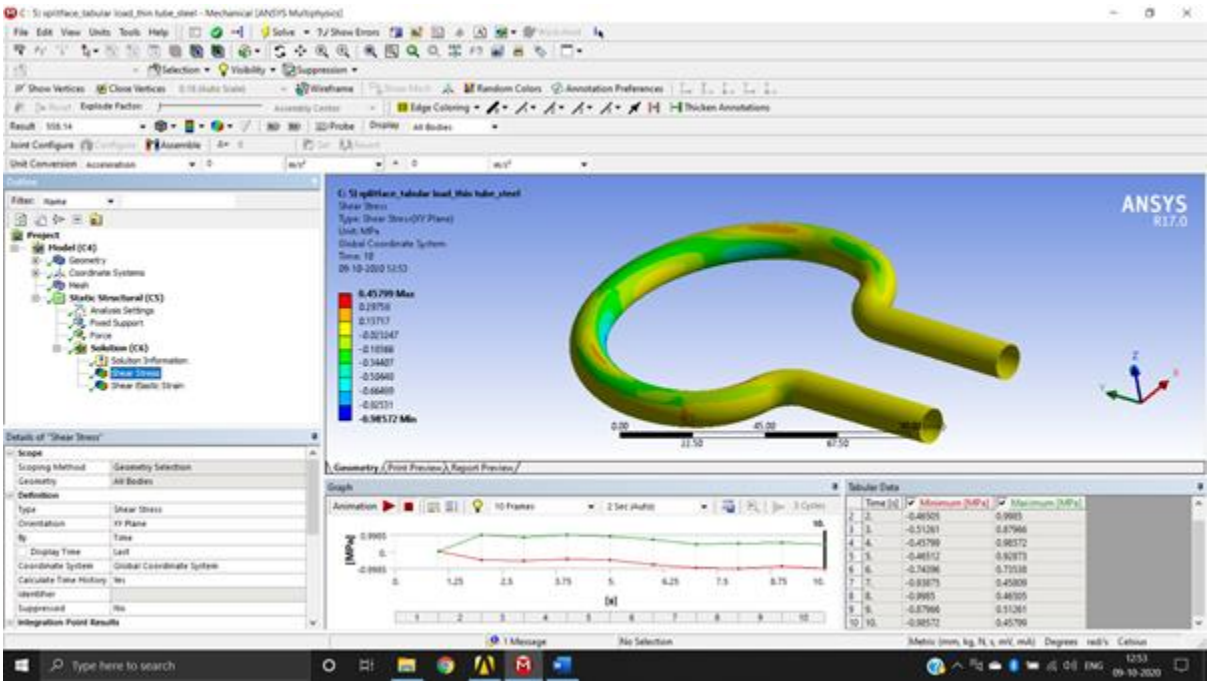
- Fixed support
- Compressive force 10 N at 4RPM



Fixed support



Force of 10N at 4RPM

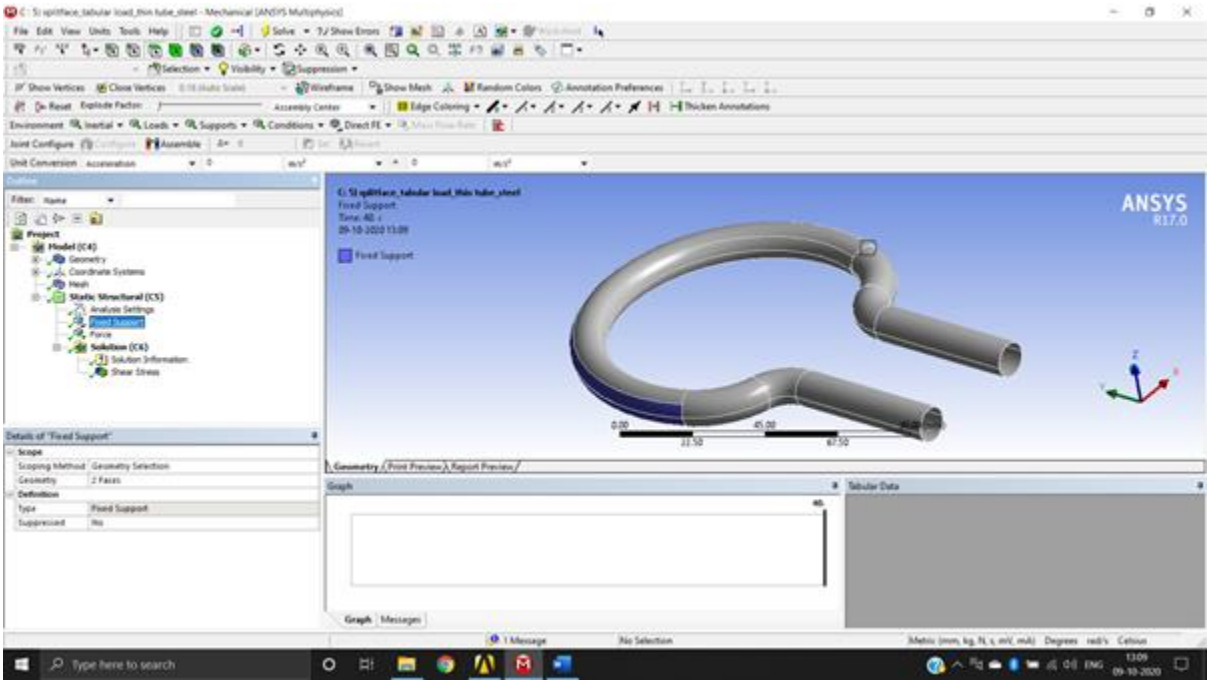


Shear stress = 0.98 MPa

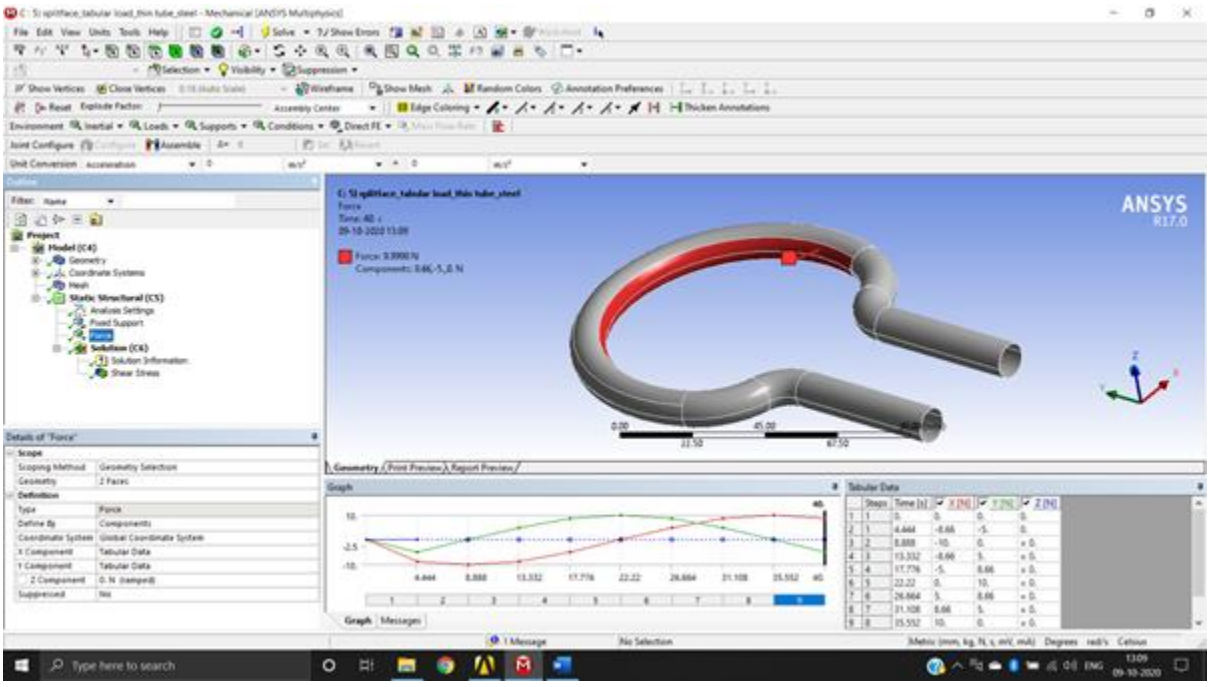
CASE 2: Tube Dimensions OD=11.4mm ID=11mm

Boundary conditions

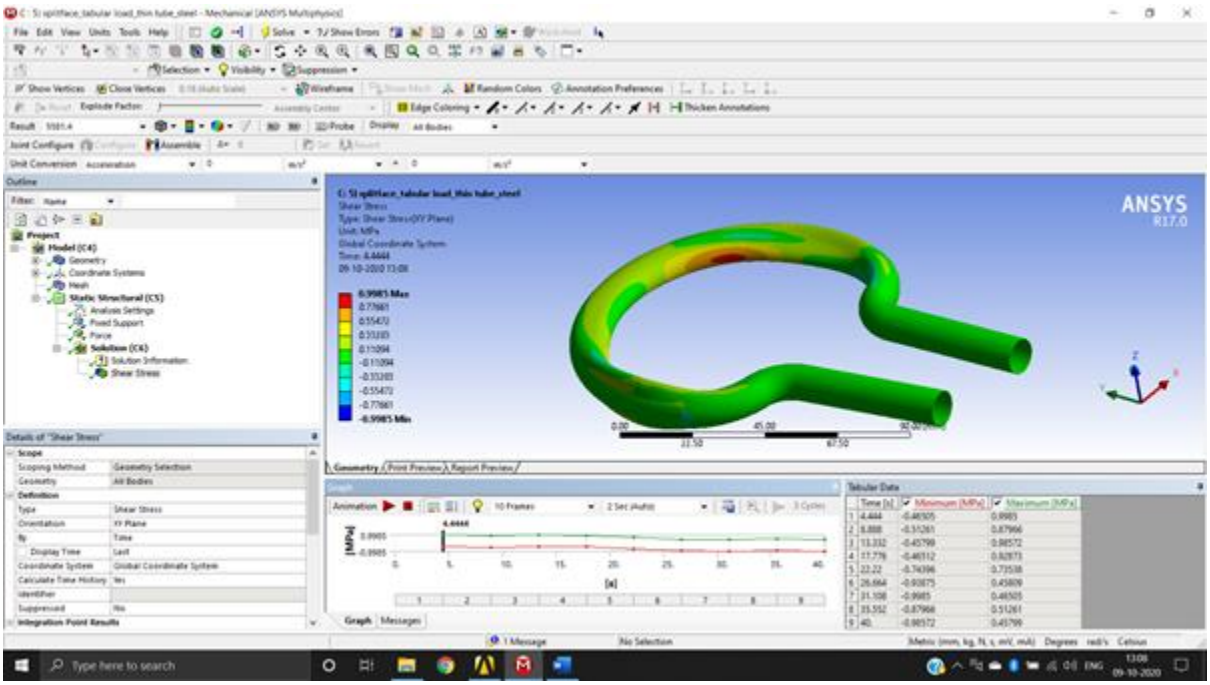
- Fixed support
- Compressive force 10 N at 1RPM



Fixed support



Force of 10N at 1RPM



Shear stress = 0.98 MPa

CONCLUSION:

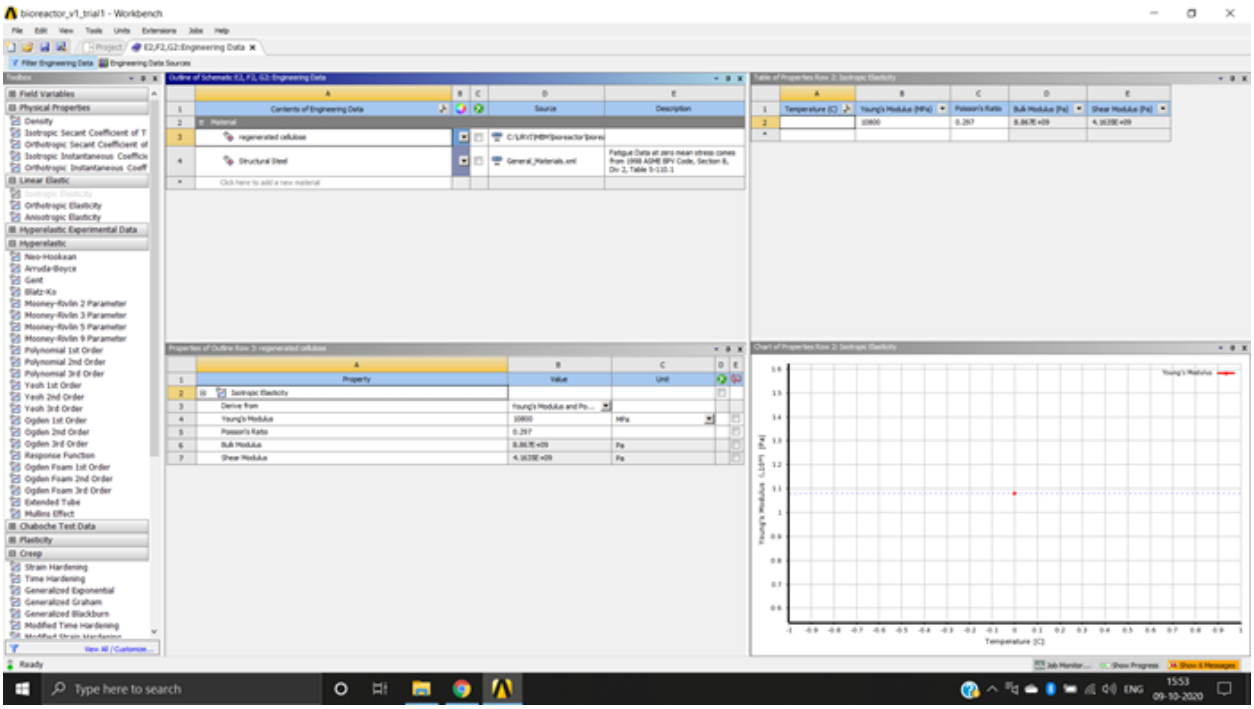
Since shear stress value obtained for the two cases (keeping all parameters except RPM constant) is the same, we can conclude that RPM does not affect shear stress generated.

DEFINITION OF MATERIAL PROPERTIES FOR REGENERATED CELLULOSE

A new material is defined on the static structural workbench, with the same mechanical properties as the dialysis tube that is used in the reactor.

Young's Modulus = 10.8 GPa [1]

Poisson's Ratio = 0.297 [10]



Now our objective is to test what value of compressive force, when applied to a tube of this material, can compress the tube to more than 50% of its initial diameter so that peristalsis occurs. The force value thus obtained is then applied to the tube as a time-varying load to test the shear stress generated.

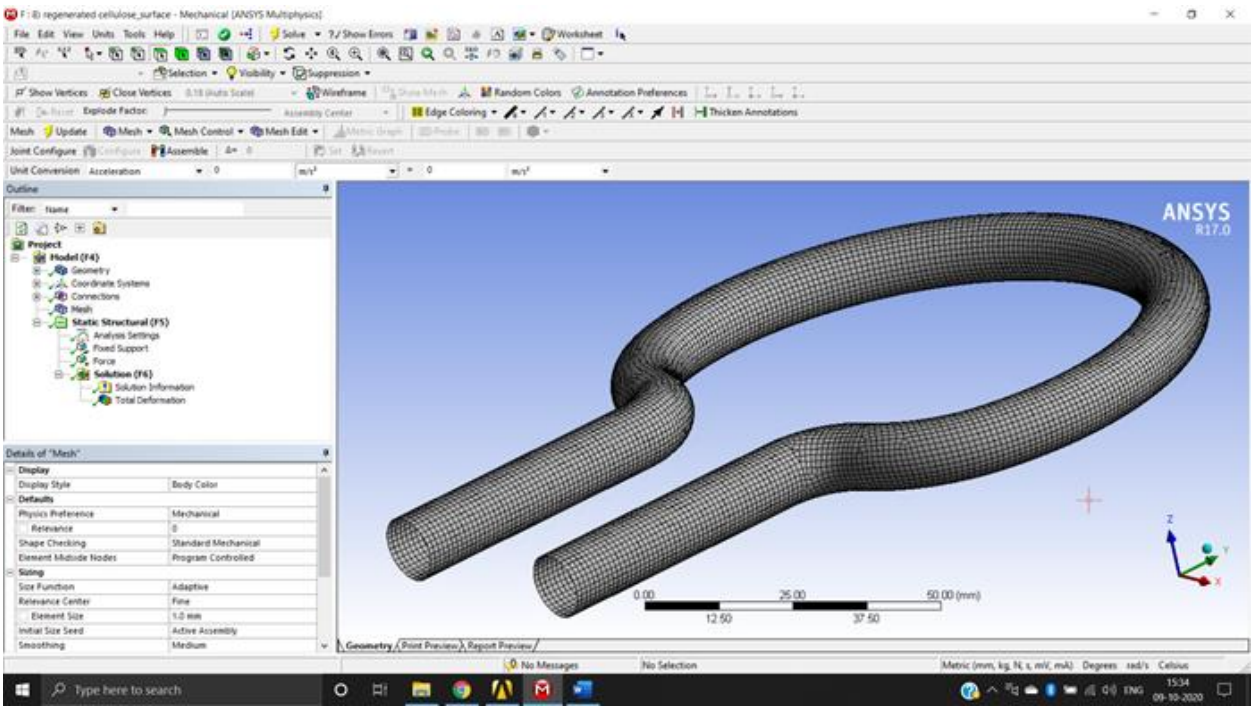
[1] (Adusumali et al., 2006) [10] (Nakamura et al., 2004)

Tube 1 (Dimensions: OD=11.4mm, thickness=15um)

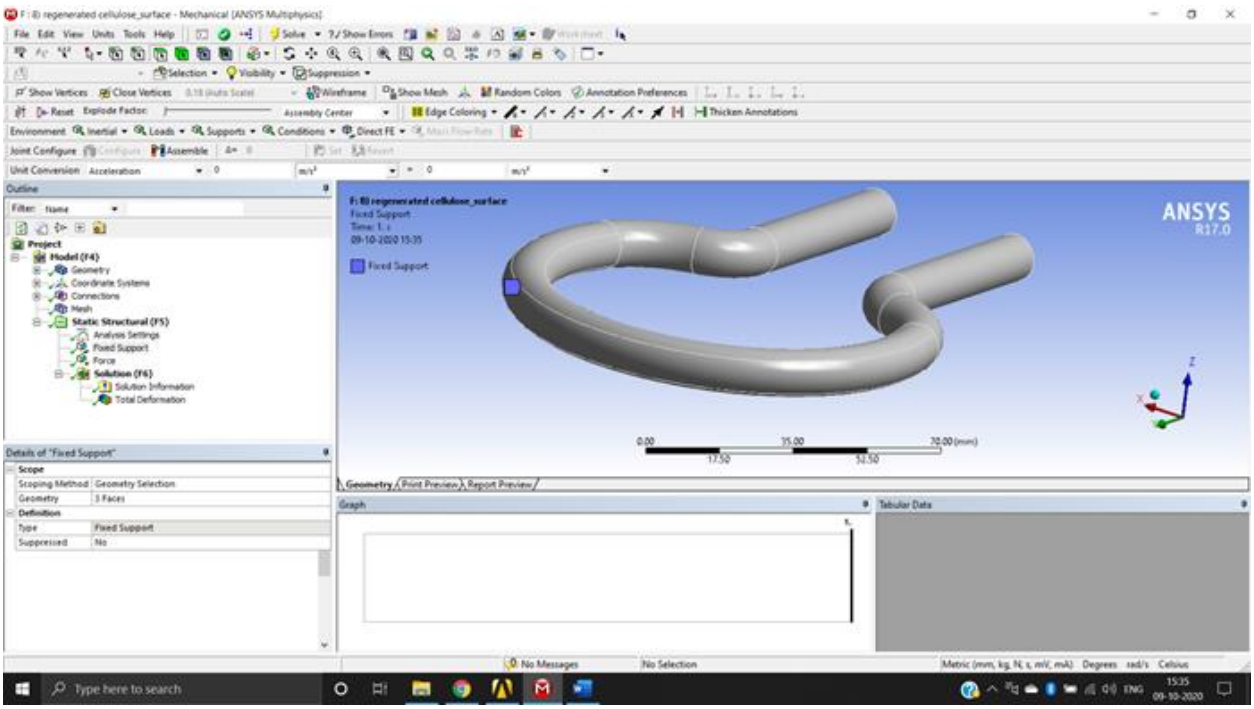
Compressive force test 1

BOUNDARY CONDITIONS

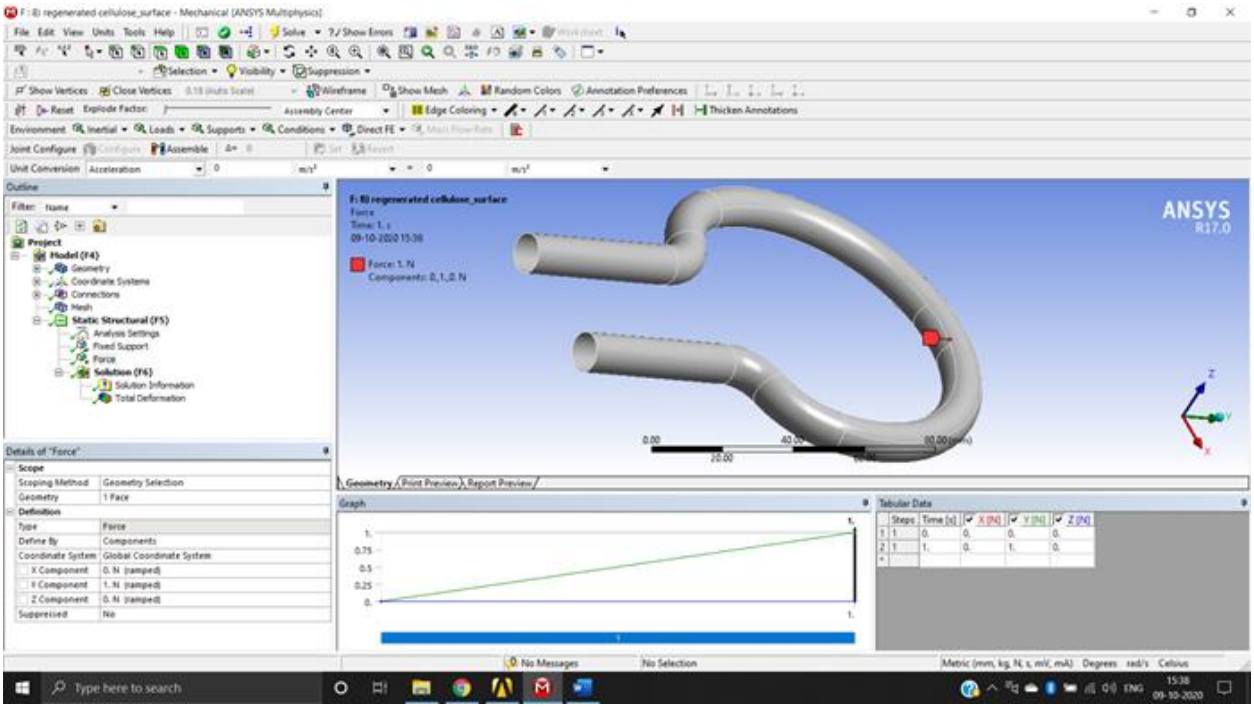
- Fixed support
- Static compressive force of 1N



Mesh is generated using adaptive size function and an element size of 1mm.



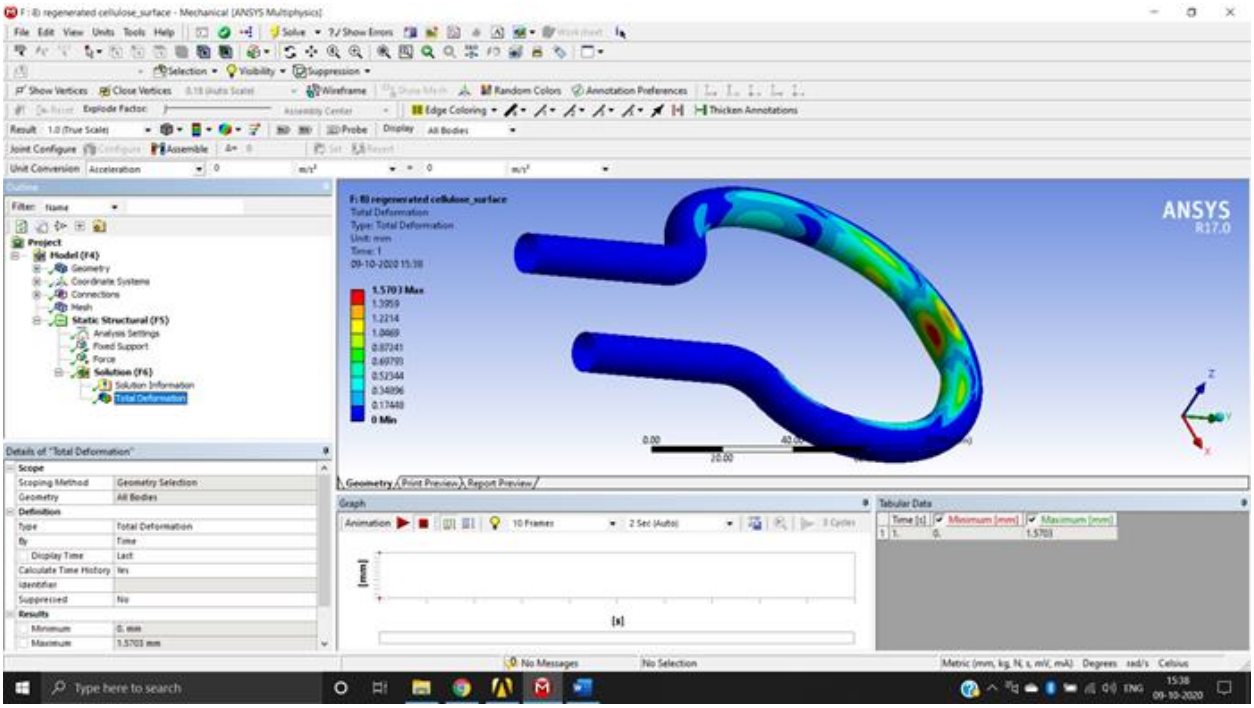
Fixed support is applied to the parts of the tube that are prevented from moving by the bioreactor case.



A static compressive load of magnitude 1N and direction along Y axis is applied to the inner surface of the tube.

RESULTS

A total deformation of 1.57 mm is obtained, which is 13.77% of the outer diameter.

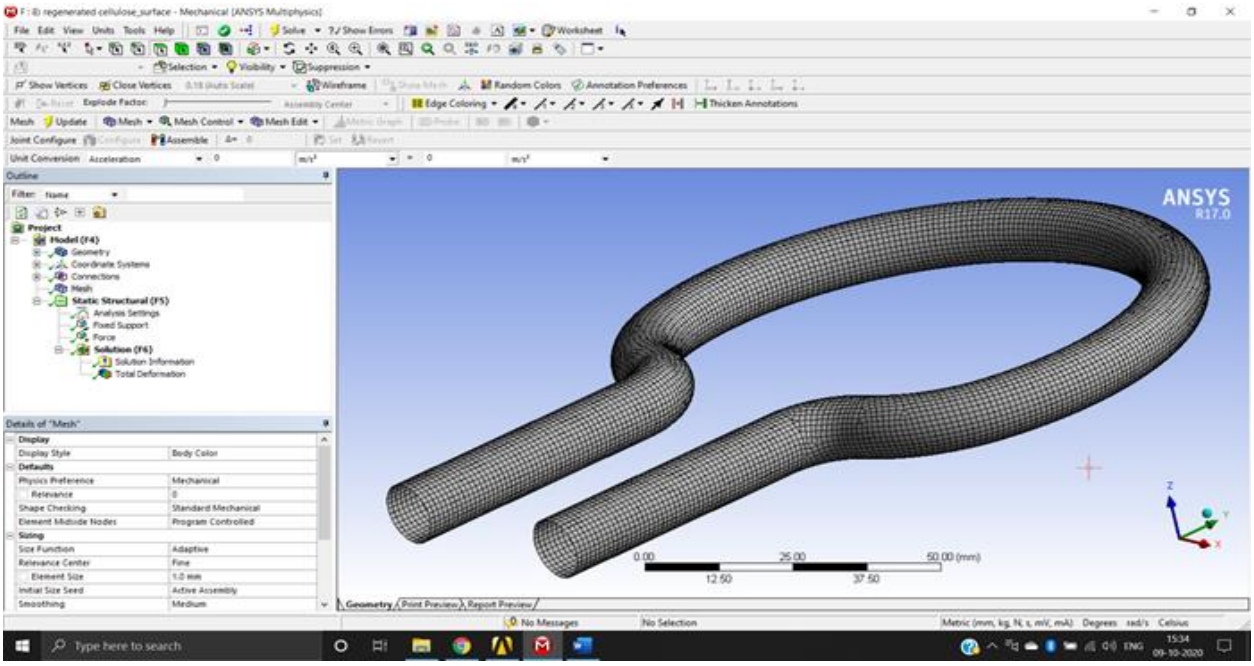


Since this deformation is very small, the test is repeated with a higher magnitude of compressive force.

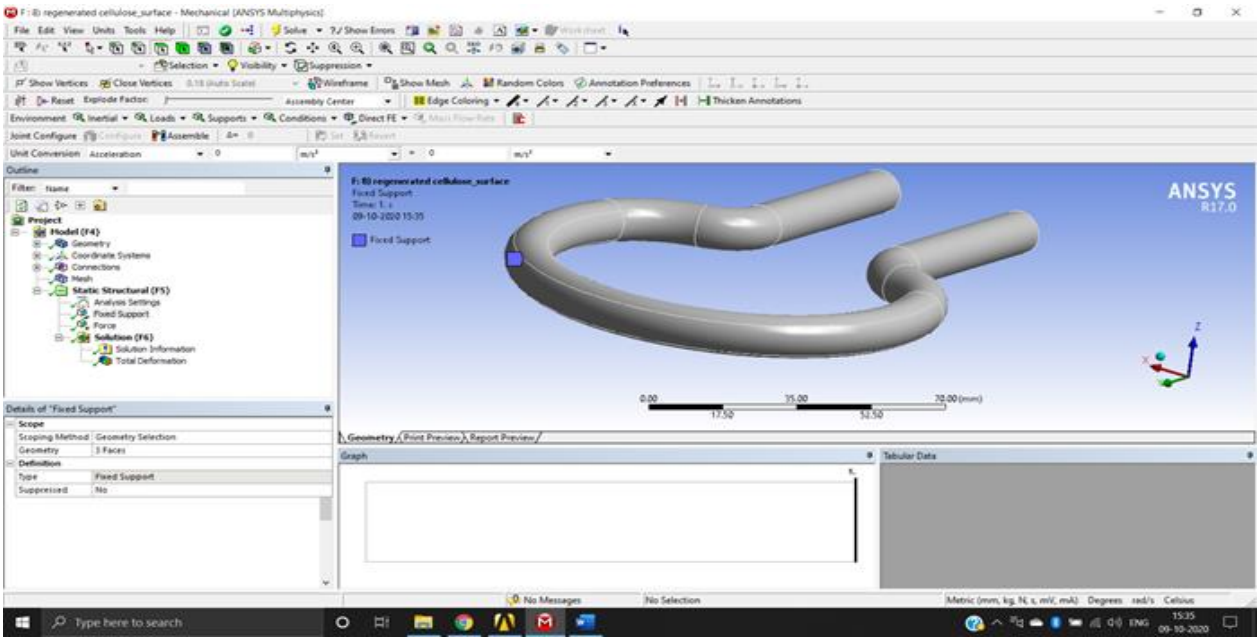
Compressive force test 2

BOUNDARY CONDITIONS

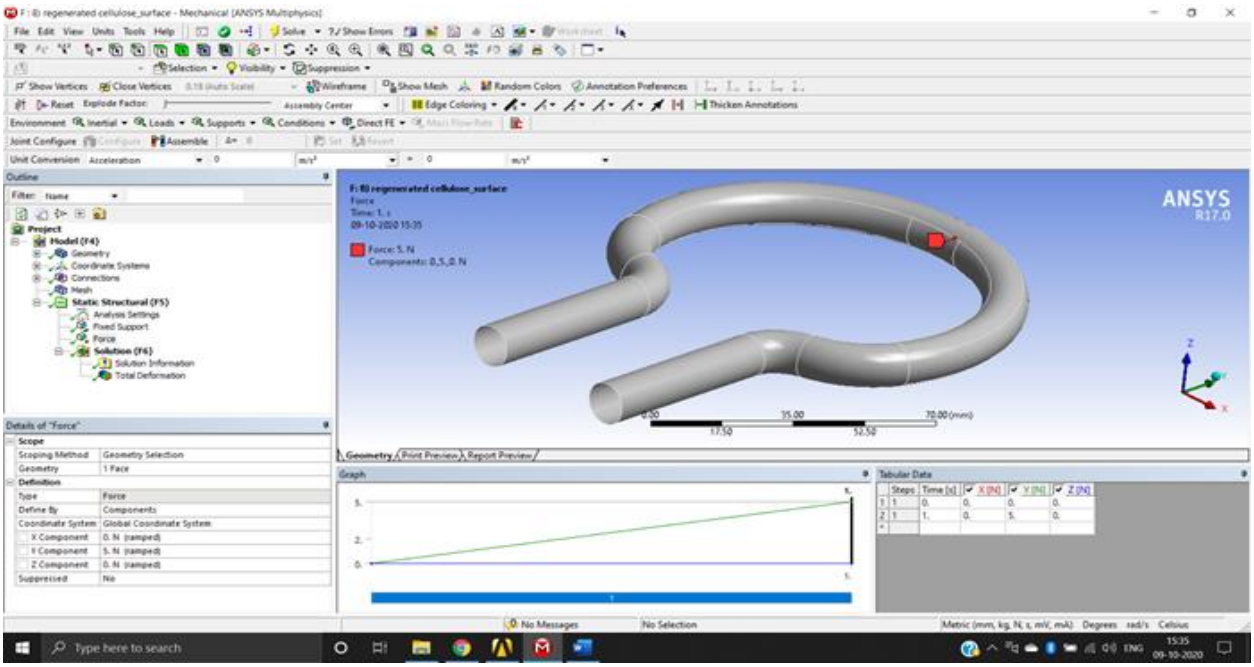
- Fixed support
- Static compressive force of 5N



Mesh is generated using adaptive size function and an element size of 1mm.



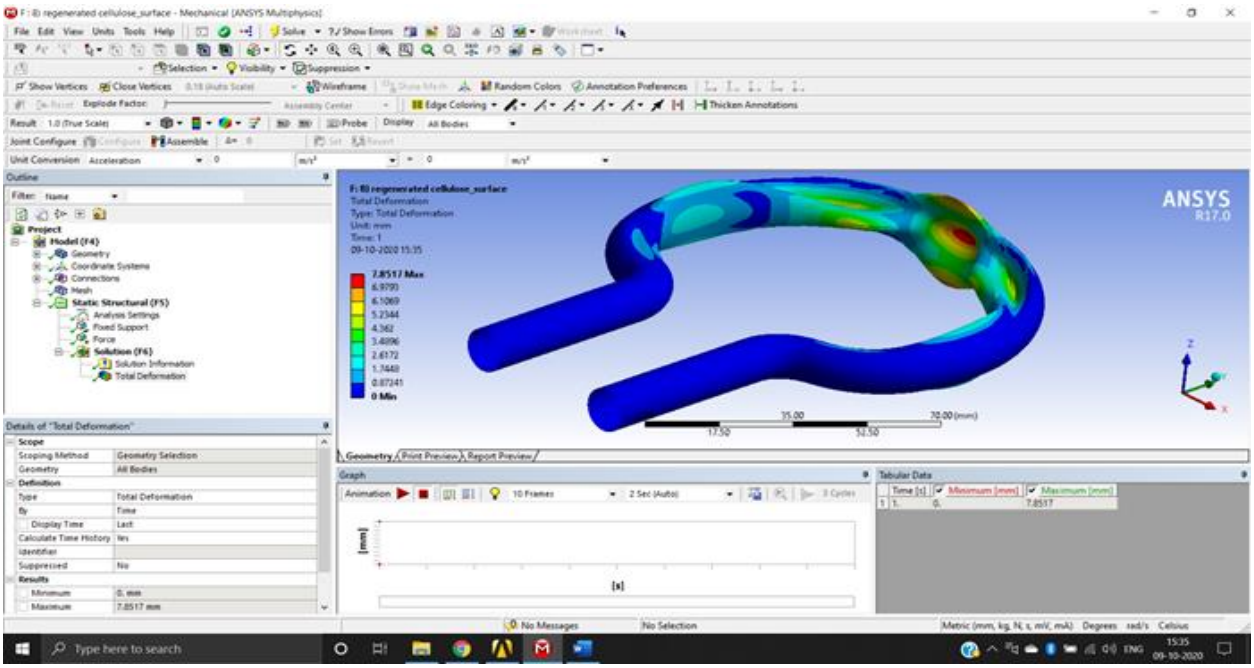
Fixed support is applied to the parts of the tube that are prevented from moving by the bioreactor case.



A static compressive load of magnitude 5N and direction along Y axis is applied to the inner surface of the tube.

RESULTS

A total deformation of 7.85 mm is obtained, which is 68.86% of the outer diameter.

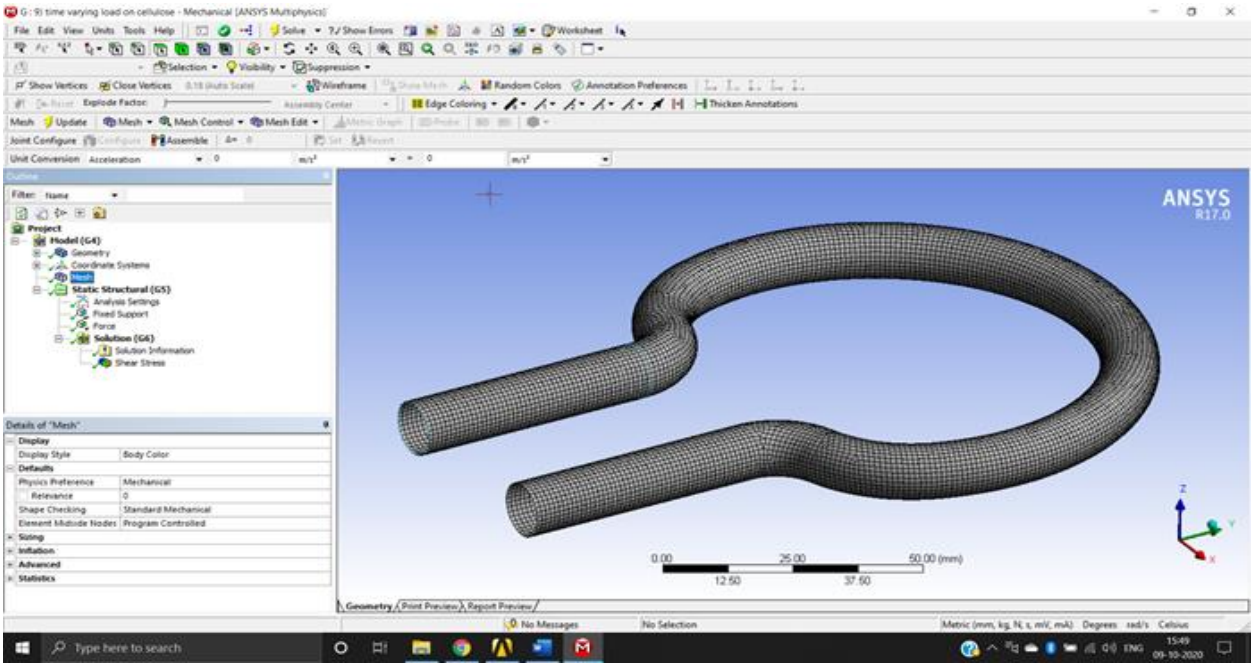


The deformation obtained is satisfactorily large. Therefore, this magnitude of force is now used as a time varying force to simulate the effect of the roller on the tube and find the shear stress generated.

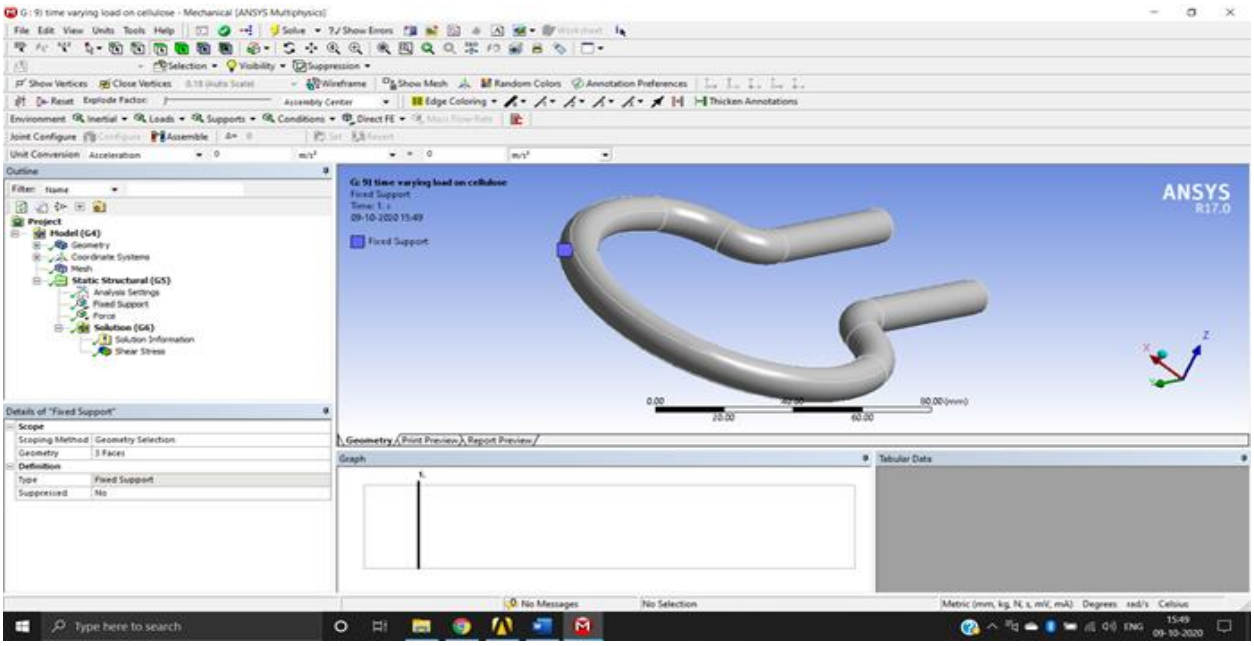
Shear stress test

BOUNDARY CONDITIONS

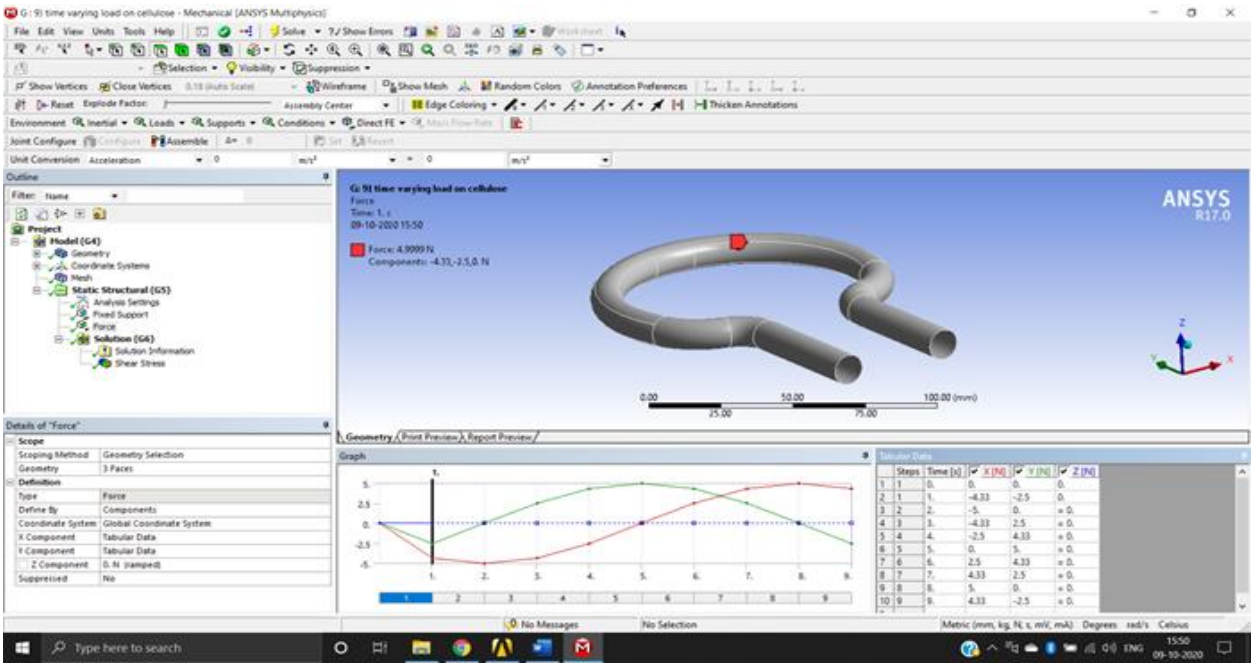
- Fixed support
- Compressive force 5 N at 4RPM



Mesh is generated using adaptive size function and an element size of 1mm.



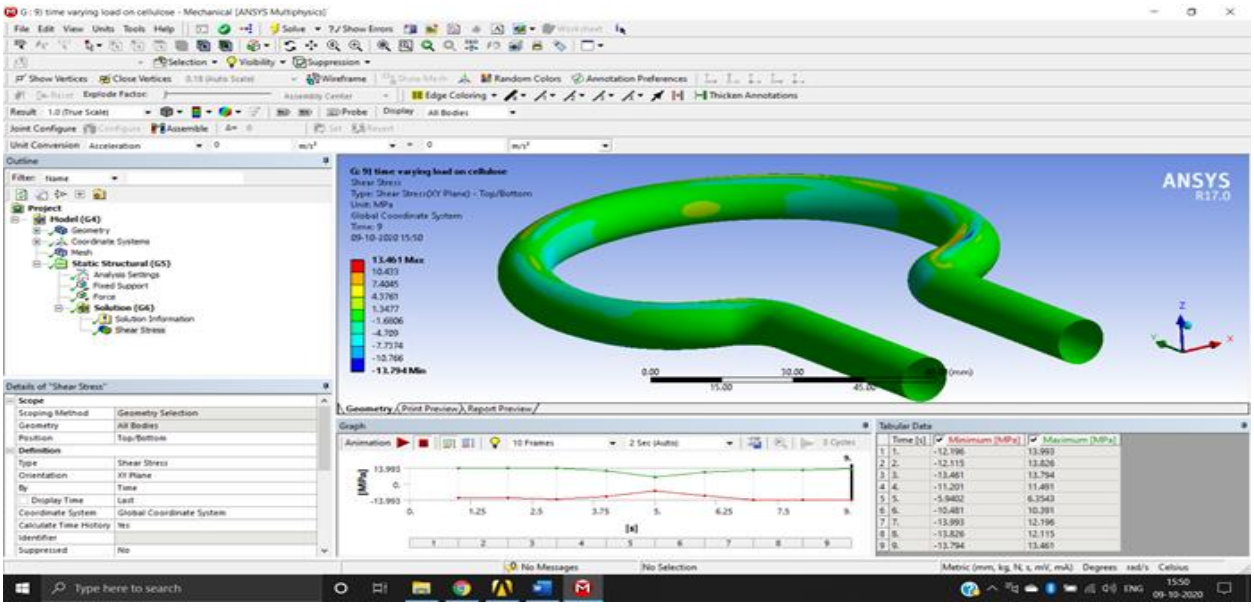
Fixed support is applied to the parts of the tube that are prevented from moving by the bioreactor case.



A time-varying compressive load of 5N is applied to the inner surface of the tube, to simulate the force applied by the roller onto the tube during peristalsis.

RESULTS

A shear stress of 13.46 MPa is obtained.



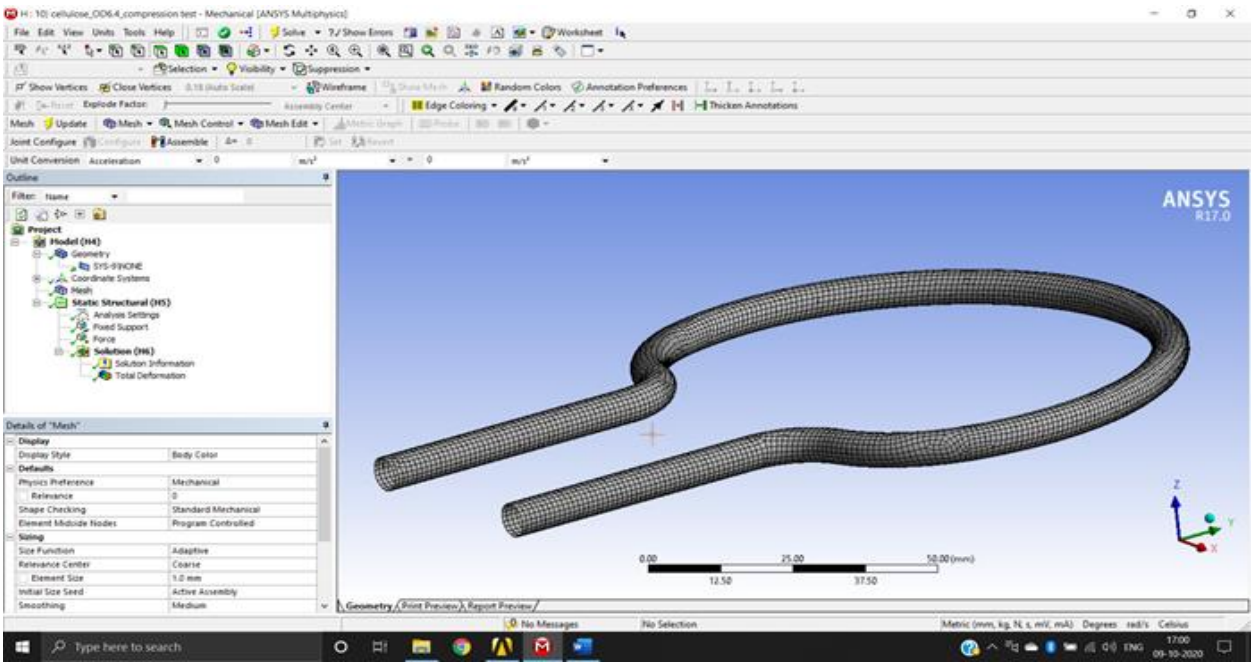
The obtained value for shear force is much larger than expected (5 orders of magnitude larger than experimentally obtained value of 200Pa). In order to closely mimic the intestinal conditions, the shear stress needs to be lowered. Thus, these tests are repeated with a dialysis tube of different dimensions.

Tube 2 (Dimensions: OD=6.4mm thickness=15um)

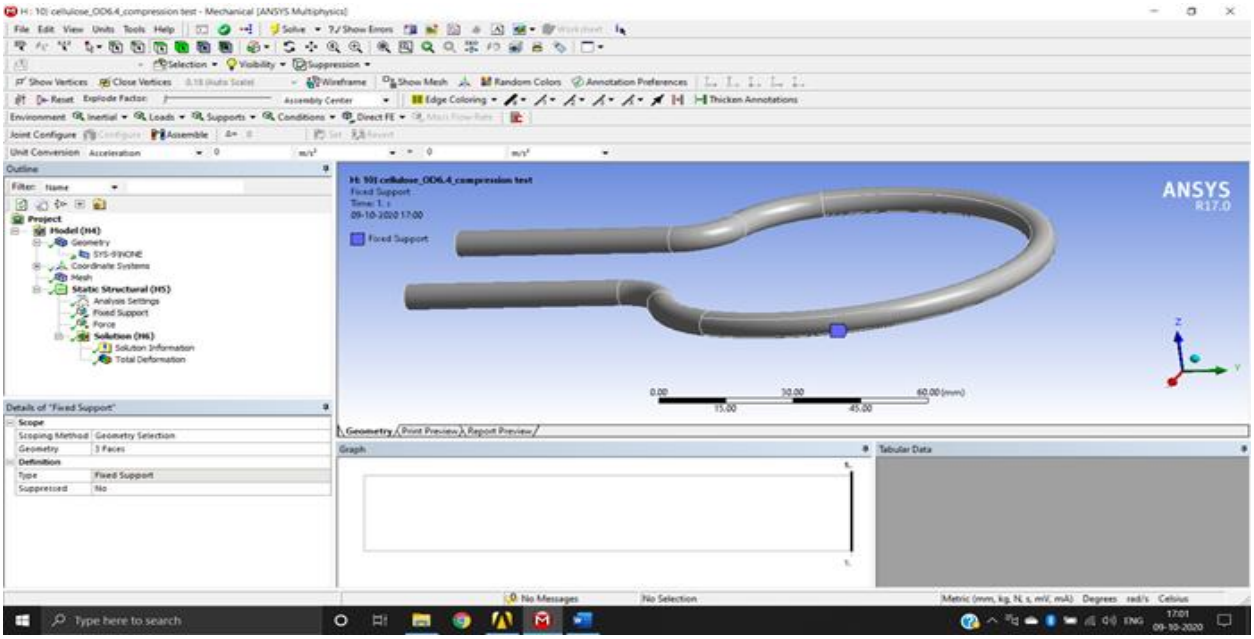
Compressive force test 1

BOUNDARY CONDITIONS

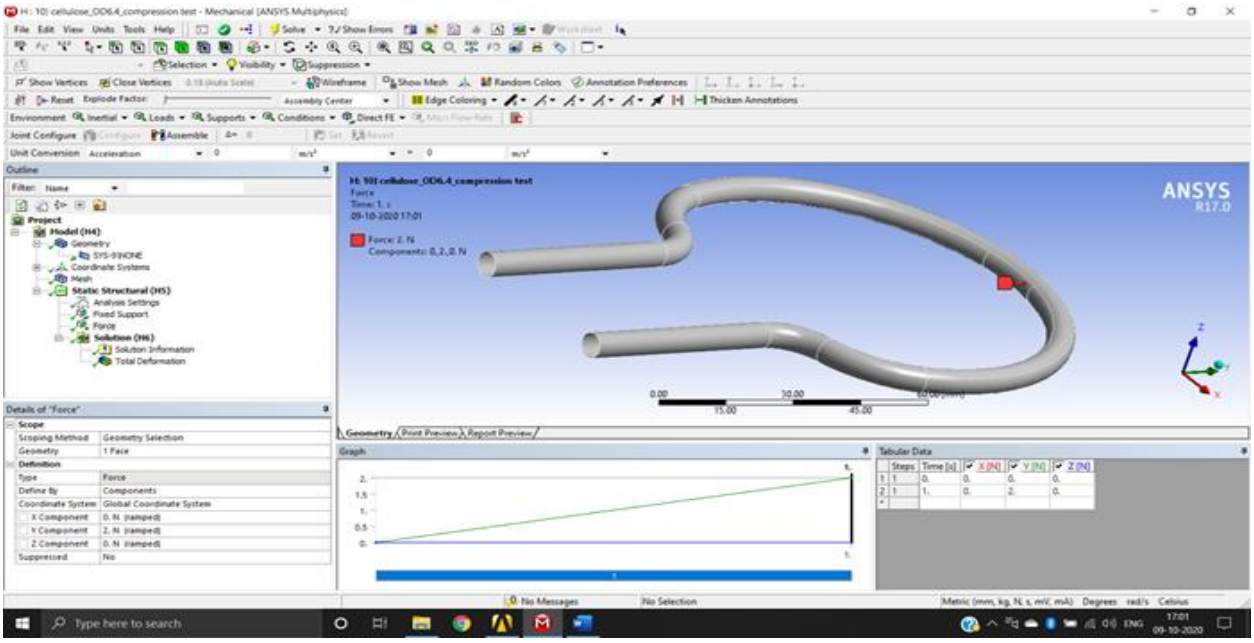
- Fixed support
- Static compressive force of 2N



Mesh is generated using adaptive size function and an element size of 1mm.



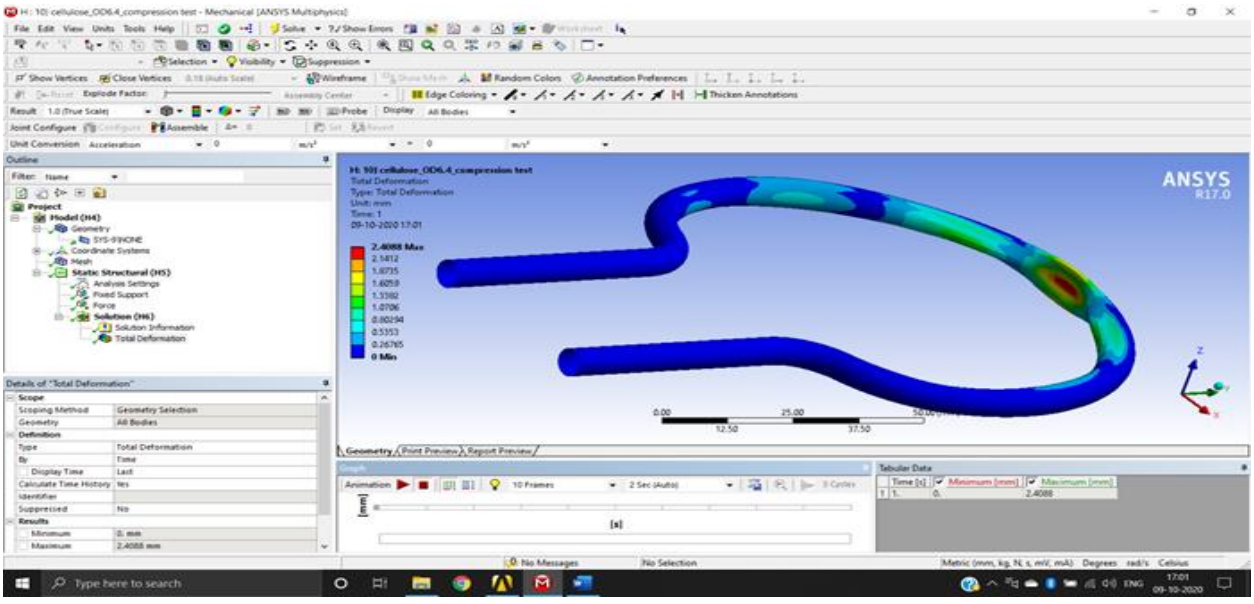
Fixed support is applied to the parts of the tube that are prevented from moving by the bioreactor case.



A static compressive load of magnitude 2N and direction along Y axis is applied to the inner surface of the tube.

RESULTS

A total deformation of 2.41 mm is obtained, which is 37.65% of the outer diameter.

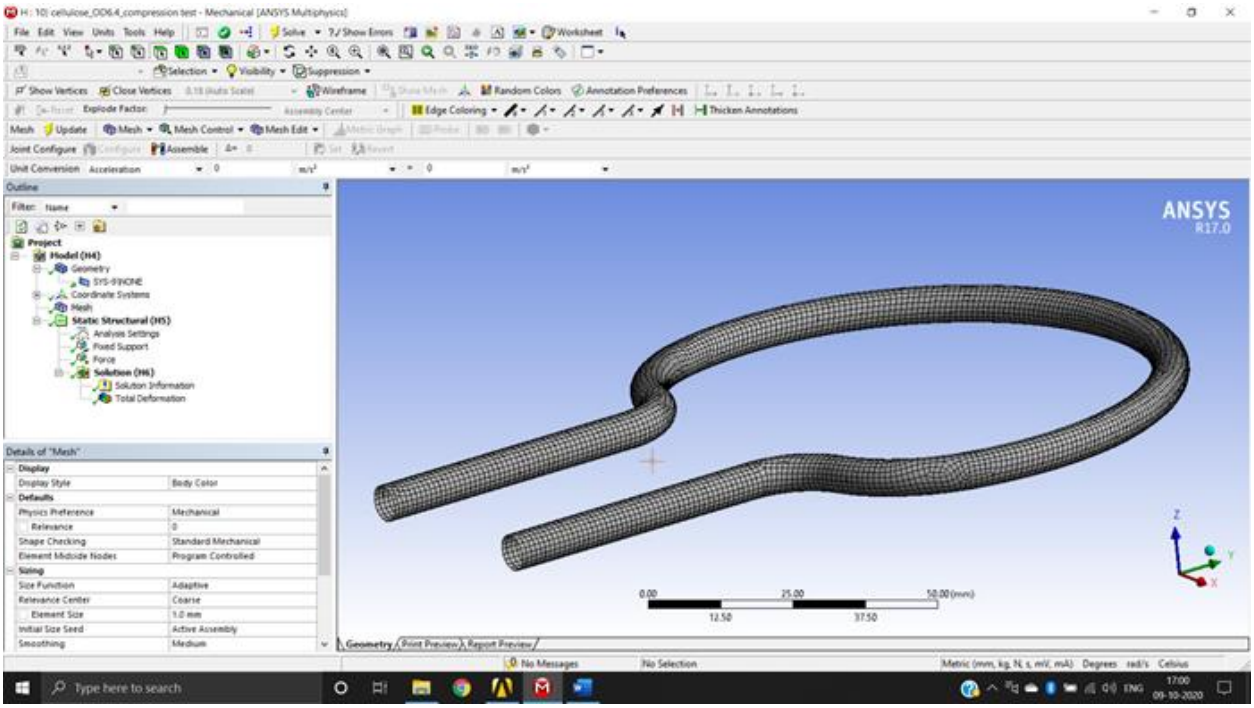


Since this deformation is very small, the test is repeated with a higher magnitude of compressive force.

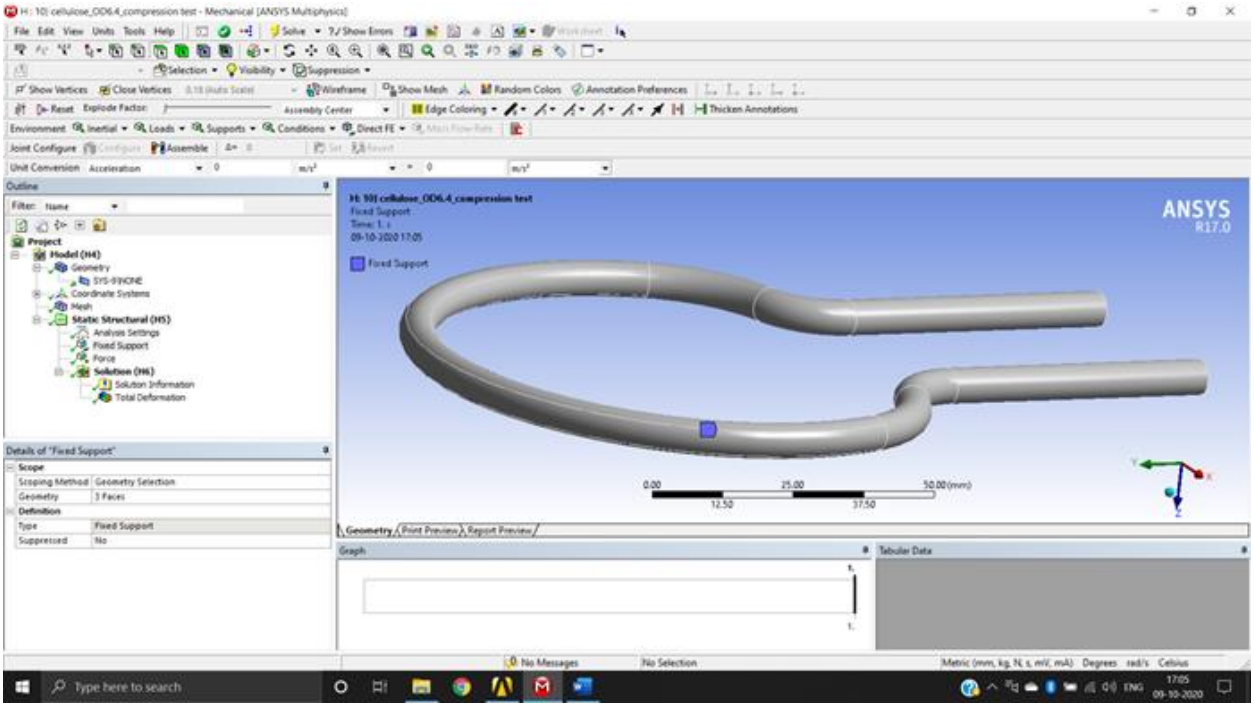
Compressive force test 2

BOUNDARY CONDITIONS

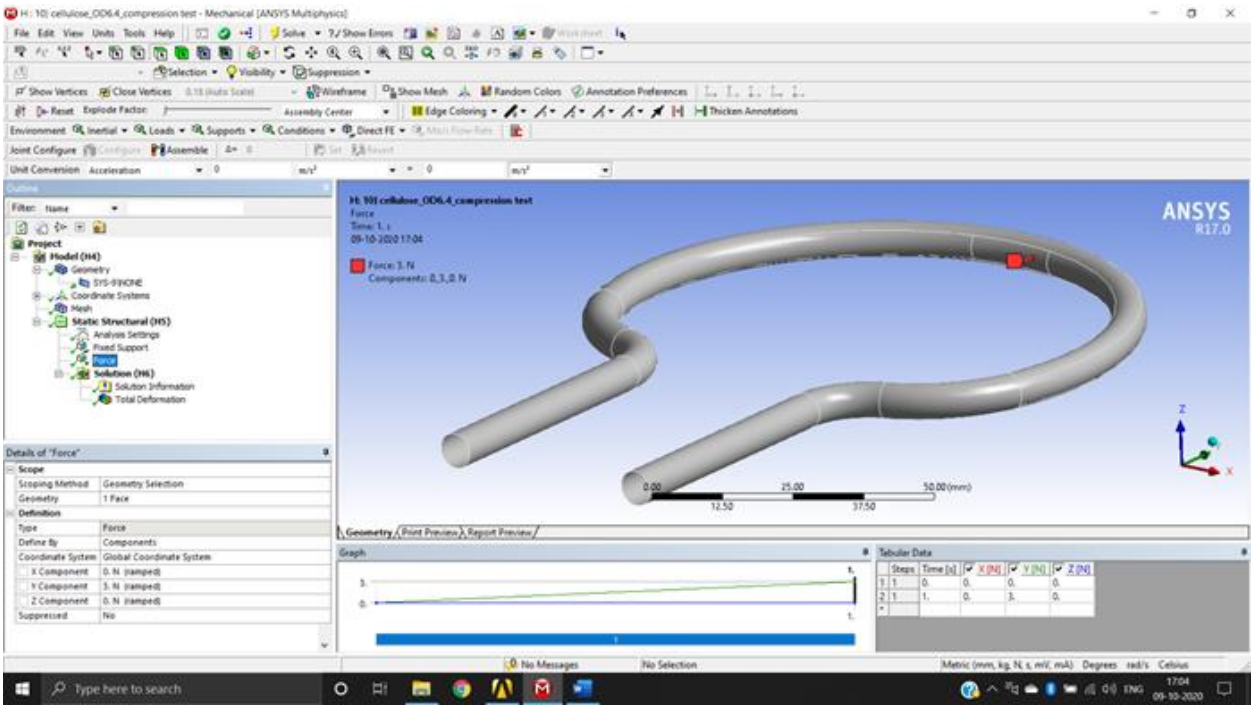
- Fixed support
- Static compressive force of 3N



Mesh is generated using adaptive size function and an element size of 1mm.



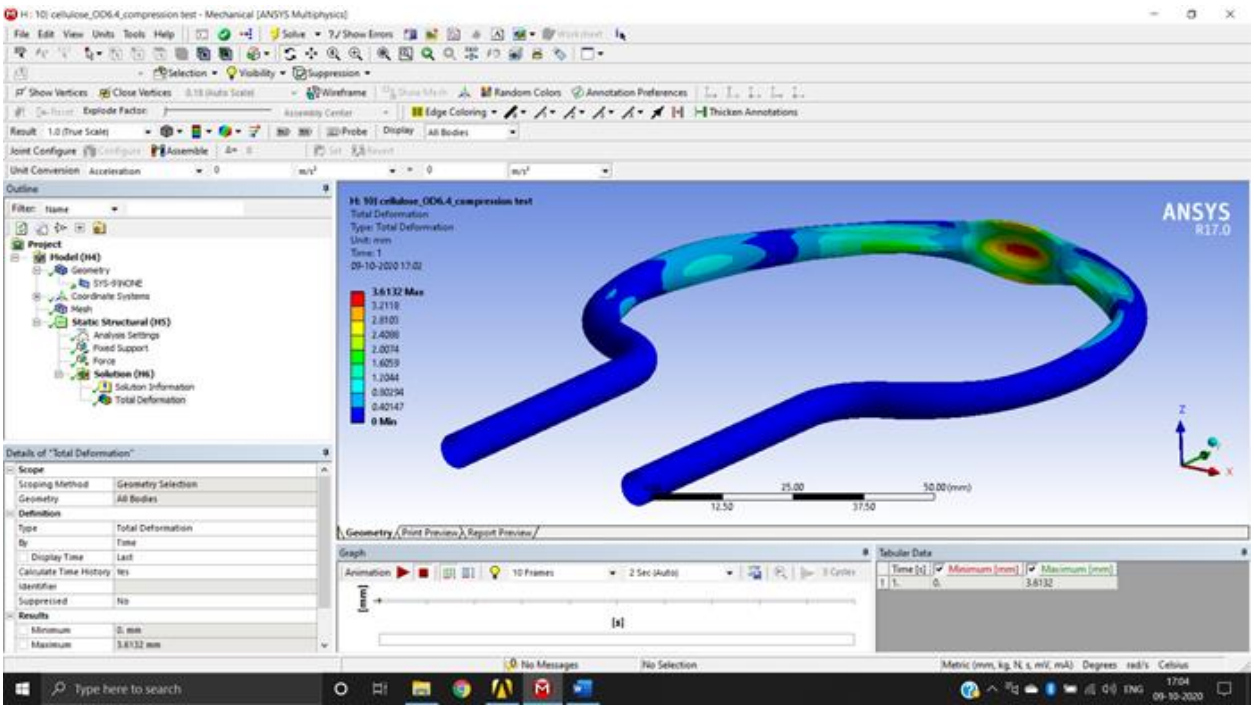
Fixed support is applied to the parts of the tube that are prevented from moving by the bioreactor case.



A static compressive load of magnitude 3N and direction along Y axis is applied to the inner surface of the tube.

RESULTS

A total deformation of 3.61 mm is obtained, which is 56.41% of the outer diameter.

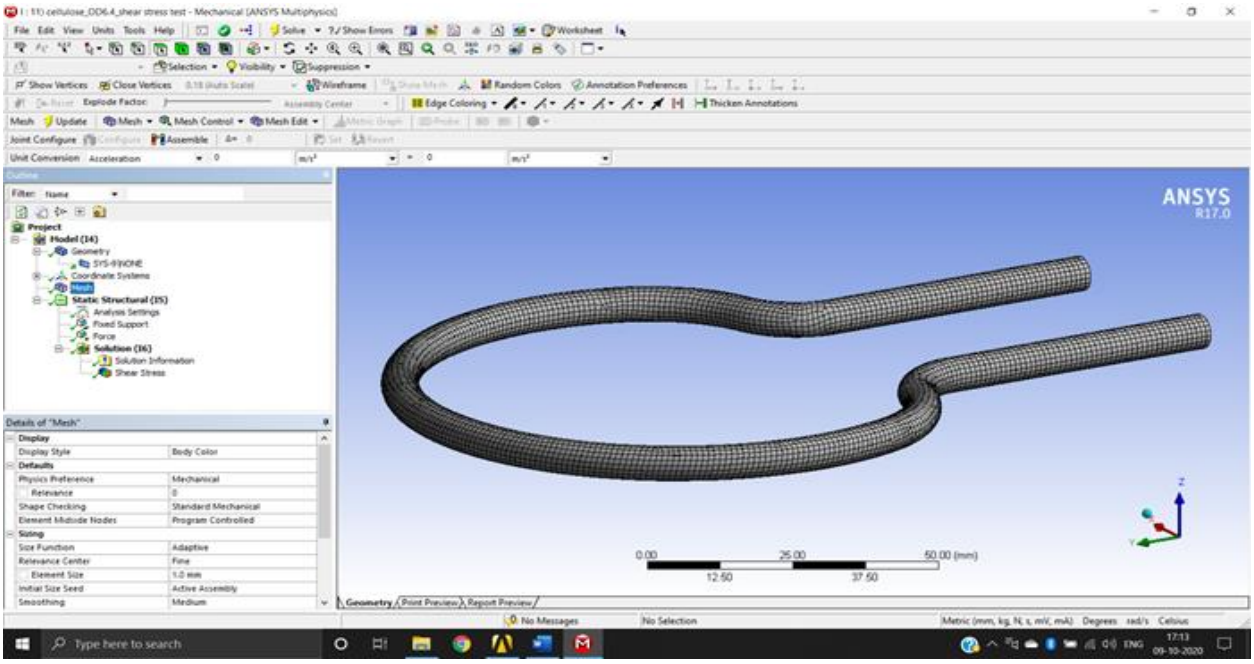


The deformation obtained is satisfactorily large. Therefore, this magnitude of force is now used as a time varying force to simulate the effect of the roller on the tube and find the shear stress generated.

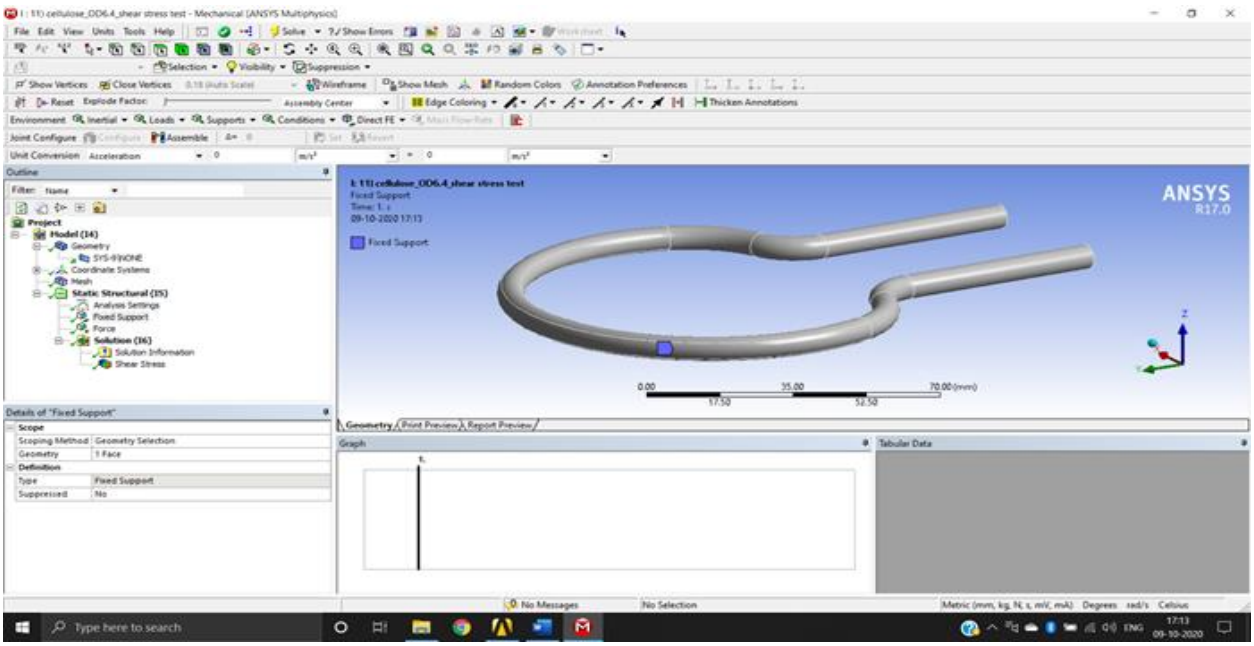
Shear stress test

BOUNDARY CONDITIONS

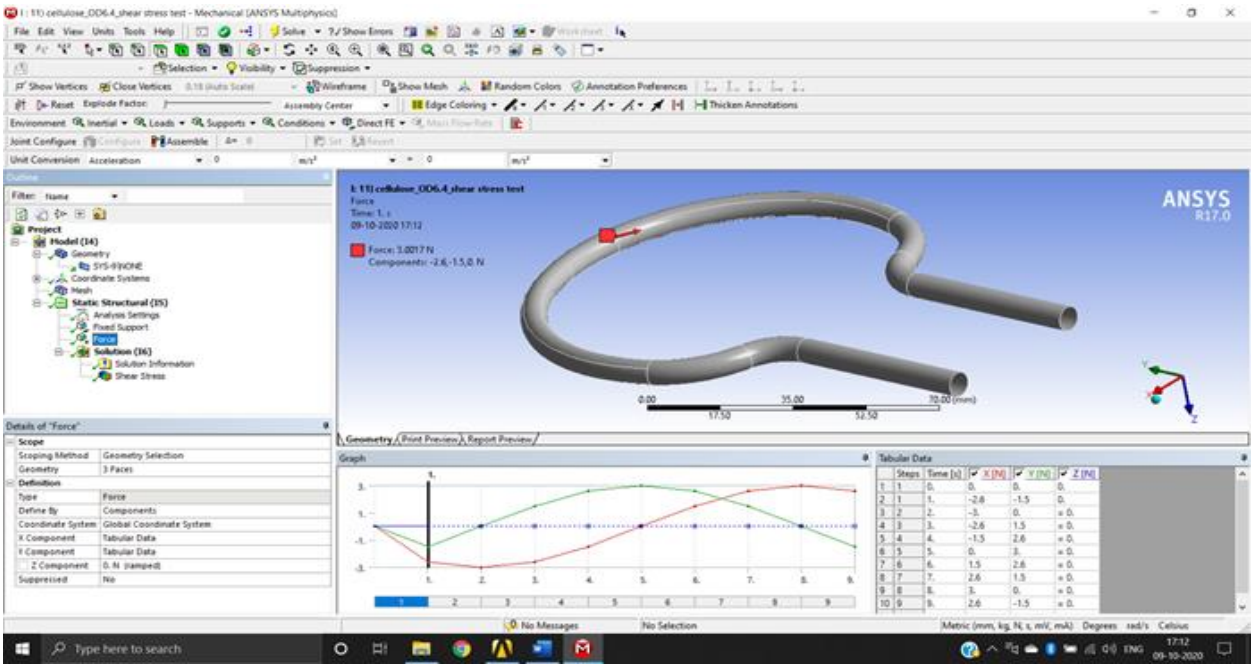
- Fixed support
- Compressive force of 3N at 4RPM



Mesh is generated using adaptive size function and an element size of 1mm.



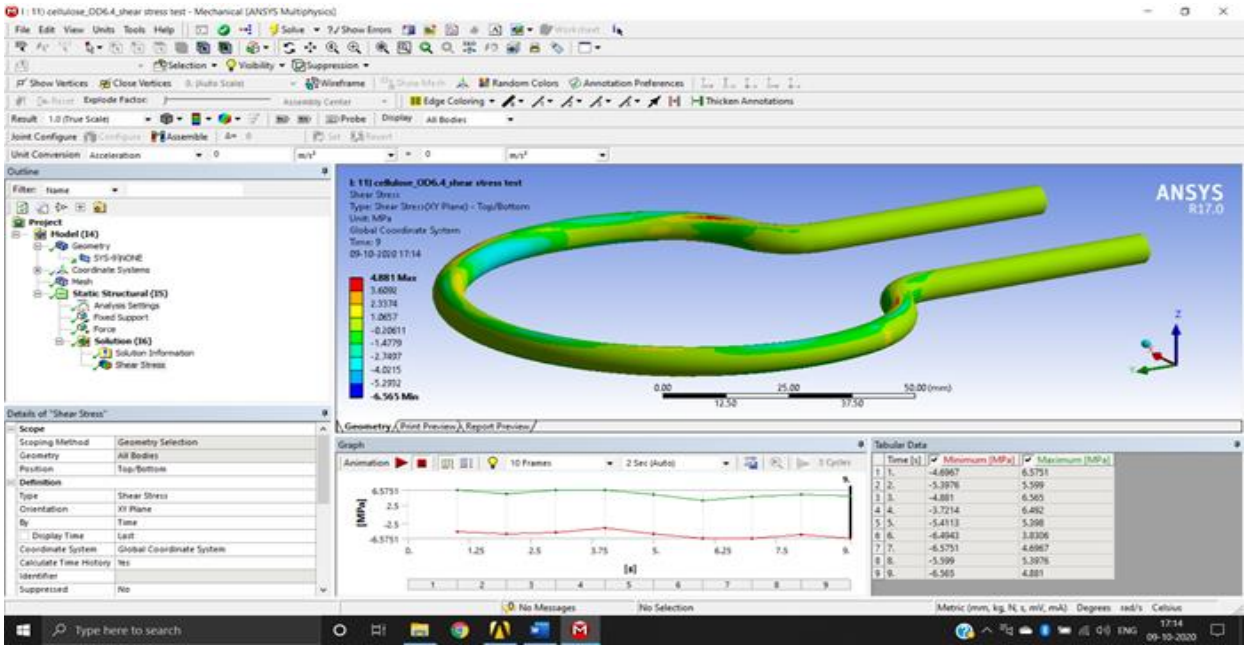
Fixed support is applied to the parts of the tube that are prevented from moving by the bioreactor case.



A time-varying compressive load of 3N is applied to the inner surface of the tube, to simulate the force applied by the roller onto the tube during peristalsis.

RESULTS

A shear stress of 4.81 MPa is obtained.



This value of shear stress is closer to expected value by one order of magnitude. It can be inferred from these tests that the shear stress would further diminish with more reduction in size of tube, but since this is the smallest available dimension, it is decided to use this tube in the bioreactor.

SUMMARY

Case	Tube dimension	Force applied	Result
Shear stress	OD 11.4mm ID 11mm	10N at 4RPM	Shear stress of 0.98 MPa
Shear stress	OD 11.4mm ID 11mm	10N at 1RPM	Shear stress of 0.98 MPa
=> RPM does not affect shear stress			
compression	OD 11.4mm Thickness 15um	1N static	Deformation of 1.57mm (13.77% of OD)
compression	OD 11.4mm Thickness 15um	5N static	Deformation of 7.85mm (68.68% of OD)
=> Compressive force of 5N is required to compress the tube to more than 50% of its original dimension => This force magnitude is used for shear stress analysis			
Shear stress	OD 11.4mm Thickness 15um	5N at 4RPM	Shear stress of 13.46 MPa
=> Shear stress obtained is much larger than expected value of around 200Pa **=> Tube of different size is analyzed to bring shear stress values closer to experimental value**			
compression	OD 6.4mm Thickness 15um	2N static	Deformation of 2.41mm (37.65% of OD)
compression	OD 6.4mm Thickness 15um	3N static	Deformation of 3.61mm (56.41% of OD)
=> Compressive force of 3N is required to compress the tube to more than 50% of its original dimension => This force magnitude is used for shear stress analysis			
Shear stress	OD 6.4mm Thickness 15um	3N at 4RPM	Shear stress of 4.81 MPa
=> New tube incurs shear stress lesser than previous results by one order of magnitude **=> Tubes of smaller diameter are not available, so this is the closest approximation possible** **=> Tube of this dimension is finalized for use in the reactor.**			

Troubleshooting of possible problems:

1. There is possibility of fluid leakage through small gaps in the model-to-motor connection or the tube-to-model connections due to imperfections is 3D printing or wear. This can be fixed by sealing the problematic areas with multipurpose sealant (M-Seal). Additionally, any leaks that do happen are contained within the incubator and can thus be cleaned up easily.
2. It is important to choose the right filament for 3D printed components of the reactor. The most commonly used filament, PLA, is not resistant to HCl (it dissolves on contact). To avoid damage to the reactor components we have used ABS filament for printing the model. ABS has excellent resistance against concentrated HCl.
3. Exposure to water and other chemicals can cause significant damage to ball bearings. The lubrication layer is prone to oxidation, and corrosion of the metal can hinder smooth rotation of the bearing. To avoid this we have used shielded ball bearings, which have a metal shield on both sides to prevent entry of contaminants.
4. To prevent back-flow of fluids from the reactor to the container bottles while the connections are being changed, a one-way valve is attached at the inlet point of every tube.
5. Isolation of all electronic components from any conducting media has to be ensured. All wiring connections should be insulated properly, and care must be taken to avoid spilling or leakage of chemicals or fluids onto the motors.
6. Since the flow rate required is lower than the rate produced by the diaphragm pump, RPM of the pump motor is reduced using PWM (pulse width modulation) to bring the flow rate down to an appropriate value.

Estimated Cost:

Component	**Rate per unit (Rs.)**	**Number of units**	**In Rupees**	**In Dollars**
ABS Filament	995/kg	0.5kg	498	6.79
Pump	260	5	1300	17.73
Valve	85	6	510	6.96
Dialysis tube	18992.42/100ft	300mm	187	2.55
Silicon tube	186.29/ft	3.28ft	608	8.29
Container bottle	830 per pack	5	830	11.32
Bearings	50	3	150	2.05
Fasteners	4	15	60	0.82
Motor	2000	1	2000	27.28
Total			**6142.04**	**83.79**

2. General Protocols for lab analysis

Note: For safety instructions of the complete handbook refer to **iGEM MIT_MAHE safety handbook**

A. Preparation of media:

i) Preparation of Luria-Bertani broth (LB broth) (1L)

Aim: To prepare a medium to grow bacteria.

Principle:

Luria-Bertani (LB) broth is the most widely used medium for the growth of bacteria. The contents are usually peptone, yeast extract and sodium chloride (NaCl). The peptone and yeast extract in the broth supply essential growth factors that the bacteria would otherwise have to synthesize. The NaCl provides essential electrolytes for transport and osmotic balance.

Materials required:

Conical flasks**,** Test tubes, Ultra-pure water, LB broth powder, Cotton, Autoclave, weighing machine, Spatula, Chloramphenicol (and/or kanamycin)- 30mg/mL in 70% Ethanol, stored at -20°C – This is a 1000X stock solution.

Procedure:

- Weigh 25g of LB broth powder and add it to 1L of ultra-pure water in an autoclavable flask.
- Swirl to mix (The powder may not dissolve completely).
- Close the flask with a cotton plug and autoclave (121°C, 20 mins).
- Cool on benchtop to about 50°C before use.
- In the laminar flow chamber, add 100 µL of the chloramphenicol (and/or Kanamycin) stock solution to 100 mL of broth. (The broth should not be too hot).
- Mix vigorously and pour it into required test tubes.

Precautions:

- The broth should not be too hot while adding chloramphenicol solution since the heat will degrade the antibiotics.

Note: **If non-selective broths (without antibiotic) are required for the experiments it would be mentioned.**

Protocol source: Luria-Bertani broth

ii) Preparation of Luria-Bertani agar (LB agar) (1L)

Aim: To prepare a medium to culture bacteria.

Principle:

Luria-Bertani (LB) agar is the most widely used medium for the culturing of bacteria. The contents are usually peptone, yeast extract, agar and sodium chloride (NaCl). The peptone and yeast extract in the broth supply essential growth factors that the bacteria would otherwise have to synthesize. The NaCl provides essential electrolytes for transport and osmotic balance. The agar is the solidifying agent that creates a gel to plate the bacteria.

Materials required:

Conical flasks**,** Petri plates, Ultra-pure water, LB broth powder, Agar, Cotton, Autoclave, weighing machine, Heating apparatus (microwave oven), Spatula, Chloramphenicol (and/or Kanamycin)-30mg/mL in 70% Ethanol, stored at -20°C - This is a 1000X stock solution.

Procedure:

- Weigh 25g of LB broth powder and 15g of agar and add it to 1000mL Ultra-pure water in an autoclavable flask.
- Microwave till the agar dissolves (Do not overheat it).
- Put the cotton plug and autoclave (120°C, 20 min).
- Cool it on benchtop and keep it in the laminar flow chamber.
- Wait until the agar is warm, but not too hot, and add the chloramphenicol (and/or Kanamycin). For 100mL of the LB agar mix, add 100µL of the 1000X stock solution.
- Swirl vigorously and immediately pour the plates - about 15 to 20mL of the mixture per plate.
- Allow the plates to solidify and store at 4°C for several weeks.

Precautions:

- The mixture should not be too hot while adding chloramphenicol solution since the heat will degrade the antibiotics.
- Do not overheat the mixture in the microwave as it will overflow.
- Pouring into plates can be done next to a flame to maintain sterility.

Note: **If non-selective plates (without antibiotic) are required for the experiments it would be mentioned.**

Protocol source: Luria-Bertani agar, Luria-Bertani agar 2

B. Polymerase Chain Reaction (PCR)

Aim: To conduct a Polymerase Chain Reaction of the DNA fragments (parts) before the Gibson Assembly Reaction.

Principle:

The principle of Polymerase Chain Reaction (PCR) is based on the temperature variations of heating and cooling-thermocycling reaction divided into three steps:

Denaturation: The hydrogen bonds between the nitrogen bases are broken and the double-stranded DNA is broken to single-stranded DNA at a high temperature. This occurs at 98°C.

Annealing: The primer (short RNA sequence) attaches to the DNA (complementary to it). The primer provides a site for the initiation of synthesis. This occurs at 60-72°C.

Extension: DNA polymerase uses the 3' end of the primer and starts DNA synthesis by adding nucleotides to the growing DNA strand. This occurs at 72°C.

These steps make up 1 cycle and each cycle is repeated for about 25-30 times resulting in the amplification of the desired DNA. This results in an exponential increase/amplification of DNA.

Materials required:

Micropipette, PCR tubes, Phusion HF DNA Polymerase, dNTP mix (10mM) (deoxyribonucleotide triphosphate), Nuclease-free Water, Insert DNA (template DNA), 10µM forward primers, 10µM reverse primers, Vortex, Ice bath, Thermal cycler, Tips, Gloves, and 5X Phusion HF Buffer.

Procedure:

Note: Maintain aseptic conditions throughout.

PCR primers used to amplify the BioBrick Parts:

Primers	Description	Sequence
VR	Reverse primer for sequencing/amplifying BioBrick parts (VR)	attaccgcctttgagtgagc (R)
VF2	Forward primer for sequencing/amplifying BioBrick parts (VF2)	tgccacctgacgtctaagaa (F)

Reaction preparation:

- Prepare a Master Mix with 10µL of 5x Phusion HF buffer, 5µL of 10mM dNTP and 2.5µL of forward and reverse primers each.
- Aliquot 8µL of the Master Mix into each PCR tube. Prepare Master Mix for 5 PCR tubes.
- Mix 2.5µL of the template DNA in 10µL of nuclease-free water.
- Take 1µL of the sample in each PCR tube with the help of a micropipette.
- Mix 2.5µL of Phusion HF DNA polymerase in 10µL of nuclease-free water, taking 1µL of the sample in each PCR tube.
- Add 15µL of nuclease-free water into each tube. Hence the total volume in each tube comes up to be 25µL.
- Mix the contents gently with the help of a vortex.

Thermocycler:

Initial Denaturation	98°C	30 seconds	1 cycle
Amplification	98°C. Primer Tm (60-70°C). 72°C	10 seconds 20 seconds 30 seconds per kb	25-30 cycles
Final Extension	72°C	5 minutes	1 cycle
Hold	4°C	-	1 cycle

Precautions:

- Proportions of different reagents should be carefully monitored to get the desired output.
- Handle with gloves and maintain aseptic conditions to prevent contamination by unwanted DNA.

Protocol source: Polymerase chain reaction

C. Construction of plasmids

i) Restriction digestion of the BioBricks using XbaI and SpeI and Agarose Gel Electrophoresis

Aim: To perform the restriction digestion (double digestion) of the amplified BioBricks with XbaI & SpeI enzymes and visualization in Agarose Gel Electrophoresis.

Principle:

Digesting a DNA substrate with two restriction endonucleases simultaneously is called double digestion. These restriction enzymes are nucleases that cleave the sugar-phosphate backbone of DNA, in a specific site called restriction site to generate a smaller set of fragments. These restriction sites are palindromic sequences. The enzyme usually cuts within the molecule and are hence called restriction endonucleases. These enzymes act as a defense system in bacteria by cleaving foreign DNA.

Restriction sites:

XbaI: 5'…T | CTAG A…3'

3'…A GATC | T…5'

SpeI: 5'…A | CTAGT…3'

3'…TGATC | A…5'

Materials required:

Distilled water, DNA (BioBricks), Control DNA, 2X Assay buffer, Restriction enzymes (XbaI, SpeI), 0.5mL Microfuge tubes (vials), Micropipettes and tips, Dry bath incubator, Vortex, Crushed ice, Beakers, Gel loading buffer and dye, Conical flask, Measuring cylinder and Staining tray.

Procedure:

- Place the vials containing restriction enzyme (XbaI and SpeI) on ice.
- Thaw the vials containing substrate (DNA) and assay buffer.
- Prepare the reaction mixture using the following constituents.

Reaction mix (XbaI and SpeI double digestion) (10 µL):

DNA (substrate)	0.5-1µg
2X assay buffer	2µL
XbaI	1µL
SpeI	1µL
Distilled water	Rest

- Gently mix by tapping. Briefly spin for a few seconds.
- Incubate the vial at 37°C for 1 hour on a dry bath.
- Meanwhile, prepare agarose gel for electrophoresis.
- After an hour, incubate at room temperature for 10 minutes.
- Add 5 µL of gel loading buffer to vials.
- Load the digested samples, 10 µL of Control DNA, and 5 µL of the gel loading dye; note down the order of loading.
- Electrophorese the samples at 50-100 V for 1-2 hours.
- Using UV illuminator visualize the gel.

Precautions:

- Make sure that the restriction enzyme does not exceed more than 10% of the total reaction volume, otherwise, the glycerol and the EDTA in the enzyme storage buffer may inhibit the digestion process.

Note: Other restriction sites that can be used are EcoR1, Pst1, and Not1 (BioBrick Compatibility).

Protocol source: Restriction digestion
For more info on BioBrick Compatibility refer here.

Agarose gel electrophoresis: Protocol

ii) Agarose Gel Electrophoresis

Aim: To visualize and analyze the restriction digested fragments.

Principle:

DNA is a negatively charged molecule at neutral pH. Gel electrophoresis apparatus is used to separate charged molecules under the influence of a constant current. The DNA molecules migrate towards the positive charge with the separation depending on the molecular size of DNA, the strength of the electric field applied, hydrophobicity of the DNA, the concentration of agarose, ionic strength of the buffer, the temperature of the buffer, and conformity of the DNA.

The agarose gel is a linear polymer extracted from seaweed. It (purified powder) is insoluble in water and buffers at room temperature but dissolves on boiling. On pouring into a mold and cooling, it undergoes polymerization acting like a molecular sieve. The DNA moves through this on the application of current. Pore size depends on the concentration of agarose used.

Small fragments can pass through the pores easily whereas the larger ones find it harder to do so and separation occurs. A visible tracking dye is applied to determine the progress of the DNA through the gel, and Serva DNA stain G is used for visualizing the DNA in the transilluminator.

Materials required:

Agarose, Micropipettes and tips, Beakers, Distilled water, 2.5X Gel loading buffer, Gel loading dye (bromophenol blue), 6X Staining dye (Serva DNA stain G), TAE 10X Buffer, Heating apparatus (microwave oven), Gel electrophoresis apparatus (Basic Unit, Gel casting tray, Gel running tray, Comb, Comb stand, Electrodes, and Power cord) and UV transilluminator.

Procedure:

Gel preparation:

- Dilute the appropriate amount of 50X TAE buffer and prepare 1X TAE.
- Prepare 1% agarose by weighing 0.5g of agarose and adding it to 50mL of 1X TAE.
- Boil the agarose in the microwave till it dissolves completely and a clear solution is obtained.
- Add 1 µL of Serva DNA stain G (staining dye) to the clear agarose solution.
- Meanwhile, place the combs of electrophoresis set approximately 2 cm away from the cathode (black).

- Pour the agarose + Serva DNA stain G solution in the central part of the tank when the temperature reaches approximately 60°C. Make sure no air bubbles are generated. The thickness of the gel should be around 0.5 to 0.9 cm.
- Keep the gel undisturbed at room temperature for the agarose to solidify.
- Pour 1 X TAE buffer into the gel tank till the buffer level stands at 0.5 to 0.8 cm above the gel surface.
- Gently lift the combs, ensuring that wells remain intact and no air bubbles are formed.

Ladder & samples:

- Mix 2 µL of the control DNA with 2 µL loading dye (bromophenol blue) and 1 µL Ultra-pure water for the preparation of the ladder.
- Mix 10 µL of PCR samples with 2 µL loading dye.

Electrophoresis conditions:

- Move the solidified gel to the electrophoresis set up and connect the wires appropriately (Red (anode), Black (cathode)).
- Load the samples (and ladder) in each well carefully and run it for 90 minutes at 100V.
- Visualize the sample in UV transilluminator.

Precautions:

- The comb/well side should face the cathode (Black).
- Make sure there aren't any air bubbles when the agarose is in its liquid form.

Protocol source: Agarose gel electrophoresis

iii) Gibson Assembly Protocol (E5510)

Aim: To ligate and assemble one or more inserts into a vector to create a circular vector containing the inserts of interest.

Principle:

Gibson assembly is a molecular cloning technique used to ligate fragments of DNA and is named after its creator Daniel G. Gibson.

Three enzymatic activities: exonuclease, polymerase and ligase are carried out in isothermal conditions. The 5' exonuclease generates long overhangs by which the 5' end sequences are chewed back. This exposes the complementary sequence for annealing to which the primer attaches followed by filling of gaps of the annealed single-stranded regions by the polymerase. The nicks of the annealed and filled-in gaps are then sealed and ligated (covalently linked) by DNA ligase.

Materials required:

Milli-Q water (dH2O), Bucket with ice, PCR tubes, gBlock(s), Linearized vector, 2X Gibson Assembly Master Mix (NEB), Thermal cycler, Fiber-free tissues, NanoDrop spectrophotometer, labels (Cryo-tags), Pipettes and tips, Pre-prepared primers.

Procedure:

- Design primers to amplify fragments (and/or vector) with appropriate overlaps.
- Determine the concentration of fragments using agarose gel electrophoresis, a Nanodrop™ instrument, or another method.

Nanodrop protocol:

- Start the NanoDrop spectrophotometer and select DNA measurement as 'Nucleic Acid' in the NanoDrop menu.
- Clean the surface of the NanoDrop with dH2O and a fibre-free tissue.
- Perform calibration and blank measurement by entering one drop of 2 µL autoclaved dH2O.

- Clean the surface again with dH2O and a fibre-free tissue, place 2 µL per sample on the NanoDrop and measure the concentration. Note the concentration on appropriate labels (Cryo-tags).

- Calculate how much insert is needed for the reaction.
- NEB recommendations:
    - When 1-3 inserts are being assembled, a total of 0.02 – 0.5 pmol of DNA fragments is needed.
    - When 4-6 inserts are being assembled, a total of 0.2-1.0 pmol of DNA fragments is needed.

Formula:

pmol= (weight in nanogram) × 1000/number of bp ×650 da

Online Tools: NEBioCalculator

(***Note:*** Yields will be best when the different inserts are present in equimolar concentrations.)

- Prepare an icebox, put every material on ice.

Set up the following reaction in a PCR tube on ice:

Assembly type	**2-3 Fragment Assembly (vector + 1-2 inserts)**	**4-6 Fragment Assembly (vector + 3-5 inserts)**	**Positive Control****
Total Amount of Fragments	0.02–0.5 pmols* (x µL)	0.2–1 pmols* (x µL)	10 µL
2X Gibson Assembly Master Mix	10 µL	10 µL	10 µL
Milli-Q water	10-XµL	10-XµL	0
Total Volume	20 µL***	20 µL***	20 µL***

* Optimized cloning efficiency is 50–100 ng of vectors with 2–3 fold of excess inserts. Use 5 times more of inserts if the size is less than 200 bps. The total volume of unpurified PCR fragments in the Gibson Assembly reaction should not exceed 20%.

** Control reagents are provided for 5 experiments.

*** If greater numbers of fragments are assembled, additional Gibson Assembly Master Mix may be required.

- Incubate samples in a thermocycler at 50°C for 15 minutes when 2 or 3 fragments are being assembled or 60 minutes when 4-6 fragments are being assembled.
- Following incubation, store samples on ice or at –20°C for subsequent transformation.

- Transform competent cells with 2µL of the assembly reaction, following the transformation protocol.

Precautions:

- Reaction time less than 15 minutes are generally not recommended. Extended incubation time (up to 4 hours) has been shown to improve assembly efficiencies in some cases. Do not incubate the reaction overnight.
- The efficiency of assembly decreases as the number or length of inserts increases. Hence use the optimal amount only.
- Inserts to be assembled should not have a stable single-stranded DNA secondary structure, such as a hairpin or a stem-loop, or repeated sequences, as this would directly compete with the required single-stranded annealing/priming of neighboring assembly fragments.

Protocol source: Gibson assembly
More information- from NEB: Gibson
Transformation: Protocol
Nanodrop: Nanodrop User Manual

D. Competent cell preparation

Aim: To prepare fresh competent cells of *Escherichia coli DH5alpha.*

Principle:

Competent cells are cells that have altered cell walls that allow DNA to easily pass through. The most widely used method to artificially induce competency in cells is the calcium chloride heat shock method. The exact mechanism of how this process works is still unknown. One of the hypothesis states that the calcium ions act as a cation bridge between the negatively charged phosphorylated lipid in lipopolysaccharide (LPS) of the cell membrane and the phosphate backbone of DNA. The ice-cold $CaCl_2$ solution facilitates the binding of DNA to the surface of the cell which enters after a short period of heat shock. Competent cells are then identified by selection or screening markers such as drug resistance (Chloramphenicol in our case) etc.

Materials required:

Petri plates with LB, LB liquid medium, Wild type *Escherichia coli Nissle 1917,* Incubator, Laminar flow chamber, Autoclave, Micropipette, tips, Sterile loop, 500mL flask, Shaker incubator, Ice, $CaCl_2$ solution, Glycerol solution, Centrifuge and Micro-centrifuge tubes.

Procedure:

Note: All the steps should be performed aseptically using sterile equipment and experimental conditions.

- Culture the wild type *Escherichia coli DH5alpha* on LB plate overnight (Do not add the antibiotic).
- Using a sterile loop inoculate one colony from the LB plate into 2 mL LB liquid medium. Shake at 37°C overnight (Do not add antibiotic).
- Inoculate 1 mL overnight cell culture into 100 mL LB medium (in a 500 mL flask).
- Shake vigorously at 37°C to OD600 ~0.25-0.3.
- Chill the culture on ice for 15 min. Also, make sure the 0.1M $CaCl_2$ solution and 0.1M $CaCl_2$ plus 15% glycerol are on ice.
- Centrifuge the cells for 10 min at 5000*g at 4°C.

- Discard the medium and resuspend the cell pellet in 30-40 mL cold 0.1M $CaCl_2$. Keep the cells on ice for 30 min.
- Centrifuge the cells as above.
- Remove the supernatant and resuspend the cell pellet in 6 ml 0.1 M $CaCl_2$ solution plus 15% glycerol.
- Pipette 0.4-0.5 ml of the cell suspension into sterile 1.5 mL micro-centrifuge tubes

Storage:

- Prepare a glycerol solution (65% glycerol, 0.1 M $MgSO_4$, 0.025 M Tris/HCl pH 8).
- Store in 1 mL aliquots at -80°C.

Precautions:

- Stock freezing can decrease the transformation efficiency, hence if possible, prepare fresh competent cells.

Protocol source: Competent cell preparation- Link1 Link2 Link3 LB Agar: protocol LB Broth: Protocol

E. Transformation

Aim: To introduce the composite BioBricks into the competent *Escherichia coli DH5alpha* (or any other bacteria).

Principle:

Genetic alteration of a cell due to direct uptake and incorporation of genetic material from its surroundings is called Transformation. It occurs through the cell membrane. It is a key step in molecular cloning. Transformation occurs in nature due to conditions such as starvation, cell density, etc. It can also be induced in the lab. The bacteria then produce multiple copies of foreign DNA which can be utilized according to the application.

Materials required:

Competent cells (*Escherichia coli DH5α)*, Plasmid DNA, Competent cells, Sterile LB broth, LB agar plate (supplemented with chloramphenicol), Microcentrifuge tubes, L-rod, Ice bath, Dry bath or water bath, Incubator with shaker and Laminar airflow chamber.

Procedure:

Note: All the steps should be performed aseptically using sterile equipment and experiment conditions.

Use media without antibiotics.

- If the competent cells were stored, take it out of -80°C and thaw on ice (approximately 20-30 mins).
- Keep LB agar plates ready (Incubate at 37°C (optional)).
- Add 1-5 µL of plasmid DNA and 20 -50 µL of competent cells into a microfuge tube and gently mix by flicking.
- Incubate the tube on ice for 20-30 mins.
- Place the tube (1/2 to 2/3 of it) in a 42°C water bath or a dry bath for 30-60 seconds (heat shock treatment).
- Put the tubes back on the ice for 2 min.
- Add 250-1,000 µL LB broth (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 45 min.

- Plate the transformed bacteria onto a 10 cm LB agar plate containing the appropriate antibiotic (Chloramphenicol and/or Kanamycin).
- Incubate plates at 37°C overnight.

Precautions:

- If the culture volume is high, centrifuge and gently collect and resuspend the cells in a smaller volume of LB broth so that there isn't too much of this media on the plates.
- If too much media is present, the bacteria diffuse through the liquid and will not grow colonies. Invert the plates after the liquid is absorbed.
- Avoid the thawing of cells before use.
- The cells should not be kept on ice for longer than 3 hours.
- The cells that have been on ice should not be used again.

F. Method for the determination of methylmercury by Gas Chromatography

Aim: To determine methylmercury using Gas Chromatography method.

Principle:

Methylmercury bromide is extracted from the sample into toluene and is selectively adsorbed on the cysteine paper. Interfering compounds are washed from the paper with toluene. The isolated methylmercury is set free with sulphuric acid containing bromide, extracted into benzene and determined by GC. The extraction procedure results in good recovery and producibility for various biological and environmental samples, good sensitivity with a detection limit of 0.1 ng/g, avoidance of difficulties arising from emulsion formation, cleaner chromatograms and faster analysis. It is particularly suitable for determination of low levels of MeHg.

They are based on addition of acid (hydrochloric, hydrobromic or hydriodic) to a homogenized sample, extraction of the MeHg halide into an organic solvent (benzene or toluene), purification by stripping with a thiol compound (cysteine, glutathione) or thiosulphate, and re-extraction into benzene. The MeHg is then determined by GC with various detection systems (electron-capture, atomic absorption, mass spectrometry, microwave emission spectrometry). The main difficulties are the formation of emulsions (often persistent) during the extraction or stripping and loss of volatile MeHg.

Materials Required:

Reagents:

L(+)-Cysteine hydrochloride solution (1%) prepared daily in 20% sodium citrate solution, Sulphuric acid (2M) saturated with cupric sulphate, Potassium bromide (4M), Hydrochloric acid (4M), Mercuric chloride as a saturated solution in benzene, Benzene and toluene, Chromatographic grade (Merck (name of the company) methylmercury and ethylmercury standard solutions (1 mg/mL).

Note:

Dissolve 116.3 mg of CH_3HgCl or 115.4 mg of C_2H_4HgCl (Merck) in 100 mL of toluene. Make working standard aqueous solutions of methylmercury and ethylmercury by appropriate dilution with benzene to cover the range 0.02-0.10 ng/µL. Dissolve the same amounts of CH_3HgCl and C_2H_4HgCl as above in 1-2 mL of acetone and dilute to 100 mL with 0.1 M hydrochloric acid. The stock and working standard solutions must be kept in darkness to prevent decomposition of MeHg by ultraviolet light.

Apparatus:

Gas chromatograph: A Hewlett Packard model 5890 instrument connected to an HP 3390. An integrator is used, with an electron capture detector (63Ni radioactive source). The chromatographic conditions are as follows:

- Column temperature: 160°C
- Injector temperature: 190°C
- Detector temperature: 280°C

Carrier gas: N_2 with a flowrate of 60 mL/min.

Glass columns: Length 1.6 m, inner diameter 2 mm, packed with 5% DEGS-PS on Supelcoport 100-120 mesh (commercially available from Supelco).

Filter papers: A narrow strip of 2 mm in width, cut from the circumference of a circular filter paper (Schleicher and Schiill, No. 58g3, 7 cm diameter) is saturated with cysteine solution and dried at room temperature in an acid-free atmosphere, just before use. It is inserted as a spiral into the extraction tube.

Procedure:

Note: All the steps should be performed aseptically using sterile equipment and experimental conditions.

Note:

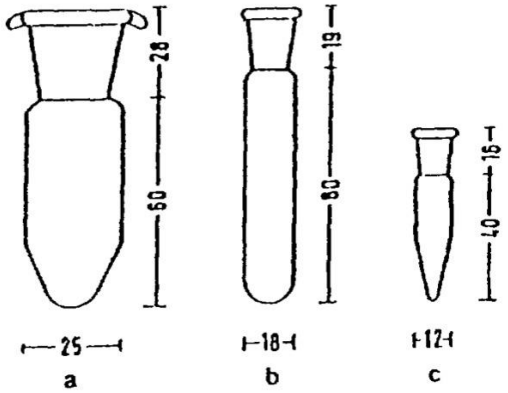
- First create a calibration curve by preparing standard solutions and measuring known concentrations of MeHg (For every experiment involving gas chromatography).

Method 1:

- Shake 1-3 g of fresh sample (0.1-1.5 g of dry sample) + 2 mL of H_2S0_4 saturated with $CuSO_4$, 2 mL of KBr solution + 3 mL of toluene in test-tube "a", Fig. 1. Equilibrate for 10 min then centrifuge for 5 min at 6000 rpm. Repeat the extraction with 2 mL of toluene and transfer the toluene phases quantitatively.
- Shake toluene phase + 1 mL of cysteine solution in test-tube "b", Fig. I. Centrifuge at 6000 rpm. Transfer the aqueous phase quantitatively. Repeat the extraction with cysteine.
- Shake aqueous phase + 1 mL of KBr solution + 1 mL of H2SO4+ 0.5-1.0 mL of benzene in test-tube "c", Fig. 1. Centrifuge.
- Transfer the benzene phase into test-tube "c", Fig. 1.
- Inject 1-5 µL of the organic phase into GC column.

Method 2:

- Shake 1-3 g of fresh sample (0.1-1.5 g of dry sample) + 2 mL of H_2S0_4 saturated with $CuSO_4$, 2 mL of KBr solution + 3 mL of toluene in test-tube "a", Fig. 1. Equilibrate for 10 min then centrifuge for 5 min at 6000 rpm. Repeat the extraction with 2 mL of toluene and transfer the toluene phases quantitatively.
- Shake toluene phase + cysteine paper in test-tube "b", Fig. 1, for 10 min. Decant organic phase and wash the cysteine paper with three 5-mL portions of toluene. Dry the paper.
- Transfer the paper into test-tube "c", Fig. 1, and add 0.1 mL of HBr + 0.1 mL of H2SO4, + 0.2-1.0 mL of benzene (0.2 mL). Equilibrate and then centrifuge.
- Transfer the benzene phase into test-tube "c", Fig. 1.
- Inject 1-5 µL of the organic phase into GC column.



Test tubes for the decomposition of samples and extraction of methylmercury: 25ml (a), 10ml (b), 3ml (c).

Fig 1: Image source

Precautions:

- It is very important to transfer only clear organic phase.
- MeHg is extracted into aqueous cysteine solution only at neutral pH.

Protocol source: Gas chromatography

G. Calibration

Aim: To calibrate instruments to ensure accurate reading

Principle:

Instrument calibration is an essential stage in most measurement procedures. It is a set of operations that establish the relationship between the output of the measurement system (e.g., the response of an instrument) and the accepted values of the calibration standards (e.g., the amount of analyte present).

Materials:

Refer here for required materials in GC, Spectrophotometer, fluorometer, 187 µg FITC, 10mL 1xPBS (phosphate buffered saline), cuvettes.

Procedure:

GFP:

Note:

- By measuring these in all standard modes in your plate reader or fluorimeter, you will generate a standard curve of fluorescence for FITC concentration.
- You will be able to use this to correct your cell-based readings to an equivalent fluorescein concentration.
- You will then be able to convert this into a concentration of GFP.

Prepare the FITC stock solution:

- Spin down FITC stock tube to make sure pellet is at the bottom of tube.
- Prepare 10x FITC stock solution by resuspending FITC in 1 mL of 1xPBS.
- Incubate the solution at 42°C for 4 hours.
- Dilute the 10x FITC stock solution in half with 1xPBS to make a 5x FITC solution and resulting concentration of FITC stock solution 2.5 µM.

[***Note:*** it is important that the FITC is properly dissolved. To check this after the incubation period, pipette up and down – if any particulates are visible in the pipette tip continue to incubate overnight.]

Note: Use 2.0mL tubes for cuvette dilutions, and then transfer 1.0mL into your cuvettes.

- Add 1 mL of PBS into tubes 2-11.
- Add 2.0 mL of FITC 5x stock solution tube 1.
- Transfer 1.0 ml of FITC stock solution tube 1 into tube 2.
- Mix tube 2 by pipetting up and down 3x and transfer 1 mL into tube 3…
- Mix tube 3 by pipetting up and down 3x and transfer 1 mL into tube 4…
- Mix tube 4 by pipetting up and down 3x and transfer 1 mL into tube 5…
- Mix tube 5 by pipetting up and down 3x and transfer 1 mL into tube 6…
- Mix tube 6 by pipetting up and down 3x and transfer 1 mL into tube 7…
- Mix tube 7 by pipetting up and down 3x and transfer 1 mL into tube 8…
- Mix tube 8 by pipetting up and down 3x and transfer 1 mL into tube 9…
- Mix tube 9 by pipetting up and down 3x and transfer 1 mL into tube 10…
- Mix tube 10 by pipetting up and down 3x and transfer 1 mL into tube 11…
- Mix tube 11 by pipetting up and down 3x and transfer 1 mL into liquid waste

Measurement:

- Measure the plate (or cuvettes) in your plate reader (or fluorimeter).
- Setup the machine with the standard GFP settings (filters or excitation and emission wavelengths) that you will use when measuring your cells (if you change them you will not be able to use this standard curve).
- Repeat the measurement several times with different settings. You will then have a series of standard curves to choose from. Most important it is necessary to use several settings that affect the sensitivity (principally gain and/or slit width).

OD_{600}:

- Calibration curve can be determined by comparing measured OD_{600} to expected OD_{600}.
- Determine the expected OD_{600} by counting cell number using an alternative technique (for example microscope slide method).
- Convert to OD_{600} using the rule of thumb that 1 OD_{600} = 8 x 108 cells/ml for *Escherichia coli*.

Note: Clumping together of cells will also affect readings, so the medium they are suspended in will also make a difference.

Gas Chromatography:

- Use known amount of Methylmercury as standard solutions following the protocol:

Concentration (µM)	**Measurement in GC (µM)**
0.00052	
0.3	
2	
5	
10	

Table 1: Calibration data for residual standard error

Conc x_i	**Absorbance** y_i	$(x_i - \bar{x})^2$	**Predicted** $\hat{y}_i = mx_i + c$	**Residuals** $y_i - \hat{y}_i$	**Residuals²** $(y_i - \hat{y}_i)^2$
$\bar{x}$	$\bar{y}$	$\sum_{i=1}^{n}(x_i - \bar{x})$			$\sum_{i=1}^{n}(y_i - \hat{y}_i)^2$

Table 2: Data required to calculate a prediction interval

If deviations are observed

- The residual standard deviation (also known as the residual standard error) is a statistical measure of the deviation of the data from the fitted regression line.

We use the equation below to calculate the residual standard deviation,

$$s(r)=\sqrt{\frac{\sum_{i=1}^{n}(y_i-\hat{y}_i)^2}{n-2}}$$

where, is the observed value of y for a given value of xi, is the value of y predicted by the equation of the calibration line for a given value of x_i, and n is the number of calibration points.

The predicted interval can be calculated using the equation,

$$s_{x_0}=\frac{s(r)}{m}\sqrt{\frac{1}{N}+\frac{1}{n}+\frac{(\bar{y}_o-\bar{y})^2}{m^2\sum_{i=1}^{n}(x_i-\bar{x})^2}}$$

where, s(r) is the residual standard deviation, n is the number of paired calibration points (x_i,y_i), m is the calculated best-fit gradient of the calibration curve, N is the number of repeat measurements made on the sample (this can vary from sample to sample and can equal 1), $\bar{y}_0$ the mean of N repeat measurements of y for the sample, $\bar{y}$ is the mean of the y values for the calibration standards, x_i is a value on the x-axis and $\bar{x}$ is the mean of the x_i values.

The uncertainty (in % relative) can be calculated using the equation mentioned below,

$$x_{pred}=\frac{\bar{y}_o-c}{m}$$

where, y_o is the mean of N repeat measurements of y for the sample, c is the intercept of the best-fit gradient of the calibration curve and m is the calculated best-fit gradient of the calibration curve.

The uncertainty in predicted values can be reduced by increasing the number of replicate measurements (N) made on the test sample.

N	S_{xo} (µM)	Uncertainty (% relative)

Table 3: Residual standard error for different values of N

Protocol source: GFP OD600 Gas chromatography Gas chromatography: Protocol

H. Measurement of CFU/mL count

Aim: To determine the growth curve of the strain at determined optimal conditions and to calculate cell density.

Principle: To ensure that the bacterium has the most suitable growth curve at these conditions, we need to study and assess its growth curve by plotting a graph of OD600 vs time.

Materials required:

Plated bacterial strain of interest, LB broth media, Non-selective agar plates (~20), Permanent marker, Spreaders, 2 mL Eppendorf tubes, Vortex, UV-VIS spectrophotometer, Cuvettes, Graphing software

Procedure:

- Revive the culture from a glycerol vial in 15 mL LB broth and incubated at 37° ± 1°C for 24h.
- Inoculate 1% (v/v) seed inoculum (6-h-old) to LB.
- Dilute the culture of bacteria 1/200 into fresh LB and shake at 30 °C for about 3 h (until OD600 ~1.0)

Note: Do not overgrow the bacteria. The turbidity of the culture should be within the linear range of the UV-VIS instrument.

- Dilute the freshly grown culture 4:1, 3:2, 2:3, 1:4, 1:9 (Culture: Media) into fresh LB media and measure the OD600 of each sample (including the original sample where OD~1).
- Dilute each of the six samples to 10^{-2} by vortexing 10 µL of sample with 990 µL of LB in a 2 mL tube; dilute the samples to 10^{-4} by vortexing 10µL of the 10^{-2} dilutions into 990 µL of LB; plate 100 µL of the 10^{-4} dilution of each sample onto a labeled non-selective agar plate.
- Dilute the solutions further by vortexing 100 µL of the 10^{-4} dilutions into 900 µL LB (to give 10^{-5}); repeat this procedure using 100 µL of the 10^{-5} dilutions into 900 µL LB (to give 10^{-6}); plate 100 µL of the resulting 10^{-5} and 10^{-6} dilutions onto non-selective agar plates.
- Incubate the plates overnight at 30 °C and count the resulting colonies.

Note: Assuming six samples are plated at three different dilutions (10^{-4}, 10^{-5}, and 10^{-6}), there should be 18 plates total.

- Calculate the CFU/mL for each of the original six samples using the equation:

CFU/mL = colonies per plate/dilution/volume plated

For example, 44 colonies from 100 µL of a 10^{-4} dilution would correspond to 4.4×10^{6} CFU/mL. Colony counts are significant if there are between 30 and 300 colonies on a plate.

- Graph data with CFU/mL on the y-axis and OD_{600} on the x-axis, setting the intercept equal to zero; add a linear trendline and record the resulting equation

Result:

- The growth rate and CFU/mL at optimal conditions can be determined.

Protocol source: CFU/mL

I. Preparation of semi-solid medium for analysis

Aim: To prepare a semi-solid food substitution for more accurate analysis

Principle: Food or Bolus is a semi-solid substance with a much higher viscosity than water. Hence to replicate the environment, this food substitute is prepared. Different materials are used to give the food different properties (Guar gum – sticky food and Carboxymethyl cellulose - fibrous food)

Materials required: Guar gum (0.1%, 0.25%, 0.4%, 0.5%, w/v) or Carboxymethyl cellulose (CMC) (0.1 and 0.5%, w/v), distilled water, Luria-Bertani Nutrient broth, magnetic stirrer and beads, beakers, weighing machine, pH probe, Viscometer.

Procedure:

Note: The amount of guar gum and CMC will vary depending on the requirements or conditions of the stomach.

Dilution media (Adjustable composition):

- Mix appropriate amounts of distilled water in different concentrations of LB broth and mix well till uniform.

Formulation 1:

- Mix the required quantities of Guar gum (depending on the viscosity required) in 1L of water and mix well with a magnetic stirrer till uniform.
- Mix the appropriate amount of mixture with 30% v/v of Luria-Bertani Broth and mix well till uniform.
- Measure the properties like viscosity and pH and note.

Formulation 2:

- Mix the required amount of CMC in 1L (Depending on the viscosity required) of water and mix well with a magnetic stirrer till uniform.
- Mix the appropriate amount of mixture with 30% v/v of Luria-Bertani Broth and mix well till uniform.

- Measure the properties like viscosity and pH and note.

Formulation 3:

- Mix the required amount of Guar Gum and CMC (Depending on the viscosity required) in 1L of water and mix well with a magnetic stirrer till uniform.
- Mix the appropriate amount of mixture with 30% v/v of Luria-Bertani Broth and mix well till uniform.
- Measure the properties like viscosity and pH and note them down.

Results

- The food substitute of appropriate properties is prepared and ready to be used for analysis.

Note: Each experiment can be carried out with all the three different formulations depending on the availability and requirement.

Protocol source: Link

3. Experimentation

A. Control experiment

Aim: To assess survival by conventional procedures.

Principle: Conventional method of bacterial assessment is to grow the cells to be studied, centrifuge and resuspend the cells in an acidic medium, with or without washing, at pH values varying between 1.0 and 3.0. Alternatively, incubating bacteria in a medium containing 0.3% Oxgall bile, porcine bile, or bile salts is also done[2] [5] [7] [10]

Materials required: Competent transformed bacteria, growth media, Bile salt solution, Spectrophotometer, incubator

Procedure:

- Prepare an inoculum containing the appropriate amount of CFU/mL.
- Incubate each inoculum in test tubes containing 9.9mL of the appropriate growth media containing 0.4% of Bile salt solution.
- Maintain the tubes in an incubator at 37°C and OD was checked at time 0, 15, 30, 45 and 90 minutes.
- Plot OD vs time graph.
- Carry out the experiment 3 times.

Note: Keep a control for this experiment (medium without bile salts).

Observation:

- The OD vs time graph is obtained

Results:

- The bacteria can tolerate bile salts and hence effectively colonize in the gut.

Note: However, there are many more different stresses encountered by the bacteria in the gut, which need to be checked.

[2] Charteris et al., 1998 [5] Jacobsen et al., 1999 [7] Lankaputhra and Shah, 1995 [11] Prasad et al., 1998

B. Capsule:

i. Capsule thickness optimization:

Aim: To optimize the thickness of the capsule used to deliver the probiotic.

Principle:

The capsule thickness contributes to the longevity of the capsule in the GI tract, especially in the stomach. However, it should not be too thick so that it can dissolve in the gut as soon as it reaches it. Thus, capsule thickness optimization is important for the drug delivery process.

Materials required:

SAUL, Capsules of different thicknesses (150um, 170um, 190um, 210um, 230um and 250um), Semi solid medium of different viscosities, bile salts, 0.1M HCl, 1M HCl, 1M $NaHCO_3$, Guar gum, distilled water, tanks (containers/bottles).

Procedure:

Tank components

Tank 1	Semi-solid medium (Full stomach), Empty stomach – Guar gum in water to make the viscosity between 10^3 Pa-s and as low as 10^{-2} Pa-s (To simulate mucous) [6]
Dry inlet	Capsule
Tank 3	0.1M and 1M HCl
Tank 4	1M $NaHCO_3$
Tank 5	4% Bile salt solution
Outlet	Mixture to be analyzed

Simulation of the stress conditions of the capsule during GI transit:

Full stomach:

- Add 100mL of 0.01 M HCl into the reactor (Similar to empty stomach) maintaining pH near 2 at the rate of 3mL/min.

- Pump 200mL of semi-solid medium into the vessel and set the rpm at 0.129 RPM (2.5ml/min) (0.261 RPM (4.0mL/min), 0.412 RPM (8mL/min), 0.618 (12mL/min), 0.773 RPM (15mL/min), 1.031 RPM (20mL/min) [11]
- Add the capsule into the device through the dry inlet.
- Add appropriate amounts of 1M HCl at 20mmol/h (reported as the maximum HCl secretion rate for human stomach (Ewe and Karbach, 1990)).

Note:

The above steps are performed with SAUL in vertical configuration (3 - 4 hours).

Empty stomach conditions will have mucous substitute instead of semi-solid medium in the inlet tank 1.

- Neutralize the contents of the device to pH 6 by adding 1M $NaHCO_3$ at a rate of 4.5 ml/min to simulate the passing of food from the stomach to the duodenum.

Note: The time taken for neutralization can vary depending on the buffering capacity of the food.

- Add 4% of bile salts solution to make the concentration in the device to 0.4% at rate of 4 ml/min during a period of 10 min.
- Dilute the bioreactor at D=0.4/h using the dilution medium to simulate the absorption of bile acids and food components in the jejunum and ileum. Maintain pH at 6.5 during the process (3 hours).
- Gradually increase the pH to 9 and reduce it to 7.4, maintain the flow at constant through the reactor (recycle stream) (3 hours).
- Plot a graph of thickness vs time.

Note: The above steps are performed with SAUL in horizontal configuration.

Observation:

- Different capsules have different tolerance to the conditions.

Results:

- Capsule thickness is optimized.

[6] Lai S. K. et al., 2009 [12] Rao S. P. et al., 2017

ii. Efficiency of acid protective coating

Aim: To check the efficiency of acid protective coating in the presence of stresses of the stomach (pH, shear stress, etc.)

Principle:

Stomach plays an important role in sterilization of food by subjecting the bacteria to high amounts of acid, due to which most bacteria are killed. If the probiotic is released into the stomach, our probiotic does not have the capabilities to tolerate such a pH condition (as demonstrated by experimentation in iGEM MIT_MAHE Composite BioBrick 2 handbook). Hence the capsule should be appropriately coated with acid resistant coating to ensure that the delivery of the probiotic occurs only in the small intestine.

Materials required:

SAUL, Capsules with different amounts of sodium alginate, hypromellose and gellan gum coating, Semi solid medium, 0.1M HCl, 1M HCl, guar gum, distilled water.

Procedure:

Tank components

Tank 1	Semi-solid medium (Full stomach), Empty stomach – Guar gum in distilled water to make the viscosity between 10^3 Pa-s and as low as 10^{-2} Pa-s (To simulate mucous) [6].
Dry inlet	Capsule
Tank 3	HCl (0.01M and 1M)
Outlet	Mixture to be analyzed

Simulation of the stress conditions of the capsule in the stomach:

Full stomach:

- Add 100mL of 0.01 M HCl into the reactor (Similar to empty stomach) maintaining pH near 2 at 3mL/min.
- Pump 200mL of semi-solid medium into the device at 0.129 RPM (2.5ml/min) (0.261 RPM (4.0mL/min), 0.412 RPM (8mL/min), 0.618 (12mL/min), 0.773 RPM (15mL/min), 1.031 RPM (20mL/min) [11].

- Add the capsule into the device.
- Add appropriate amounts of 1M HCl at 20mmol/h (reported as the maximum HCl secretion rate for human stomach (Ewe and Karbach 1990)).

Note:

The above steps are performed with SAUL in vertical configuration (4 hours).

Empty stomach conditions will have mucous substitute instead of semi-solid medium in the inlet tank 1.

- Note the time taken for the substance to dissolve and draw a graph of thickness vs time.

Observation:

- Different capsules have different tolerance to the conditions.

Results:

- Capsule acid coating (sodium alginate, hypromellose and gellan gum) is optimized, and tolerance time is determined.

[6] Lai S. K. et al., 2009 [12] Rao S. P. et al., 2017

iii. Stresses experienced by the capsule in the stomach and gut

Aim: To check the overall efficiency of the capsule by subjecting it to stresses experienced in stomach and gut (due to flow rate, temperature, pressure, pH, etc).

Principle:

Capsule efficiency is very important in the probiotic drug delivery process. During initial testing of new formulations, the efficiency of the capsule is tested through separate experiments for each of the properties. To give a combined, more dynamic analysis this experiment can be performed using SAUL.

Materials required:

SAUL, Final Capsules, Semi solid medium, bile salts, 0.1M HCl, 1M HCl, 1M $NaHCO_3$, Guar gum, distilled water.

Procedure:

Tank components

Tank 1	Semi-solid medium (Full stomach), Empty stomach – Guar gum in distilled water to make the viscosity between 10^3 Pa-s and as low as 10^{-2} Pa-s (To simulate mucous) [6].
Dry inlet	Capsule
Tank 3	HCl (0.01M and 1M)
Tank 4	1M $NaHCO_3$
Tank 5	4% Bile salt solution
Outlet	Mixture to be analyzed

Simulation of the stress conditions of the capsule during GI transit:

Full stomach:

- Add 100mL of 0.01 M HCl into the reactor (Similar to empty stomach) maintaining pH near 2 at flow rate of 3mL/min.
- Pump 200mL of semi-solid medium into the device at 0.380 RPM (2.5ml/min), 0.609 RPM (4.0mL/min), 1.218 RPM (8mL/min), 1.827 (12mL/min), 2.283 RPM (15mL/min), 3.044 RPM (20mL/min) [11].

- Add the capsule into the device.
- Add appropriate amounts of 1M HCl at 20mmol/h (reported as the maximum HCl secretion rate for human stomach (Ewe and Karbach 1990)).

Note:

The above steps are performed with SAUL in vertical configuration (4 hours).

Empty stomach conditions will have mucous substitute instead of semi-solid medium in the inlet tank 1.

- Neutralize the contents of the device to pH 6 by adding 1M $NaHCO_3$ at a rate of 4.5 ml/min to simulate the passing of food from the stomach to the duodenum.

Note: The time taken for neutralization can vary depending on the buffering capacity of the food.

- Add 4% of bile salts solution to make the concentration in the device to 0.4% at rate of 4mL/min for a period of 10 min.
- Dilute the bioreactor at D=0.4/h using the dilution medium to simulate the absorption of bile acids and food components in the jejunum and ileum. Maintain pH at 6.5 during the process.
- Gradually increase the pH to 9 and then reduce it to 7.4 and maintain the flow at a constant flow rate through the reactor (recycle stream).
- Repeat the experiment with different flow rates, temperatures and viscosities of the semi-solid medium and mucous substitute.
- Plot a graph of parameter vs time.

Note: The above steps are performed with SAUL in horizontal configuration (6 hours).

Observation:

- Different capsules have different tolerance to the conditions.

Results:

- Final capsule formulation is determined.

Note: The conventional experiments for testing the capsule would also be performed.

[6] Lai S. K. et al., 2009 [12] Rao S. P. et al., 2017

iv. Diffusion studies using a powdered dye

Aim: To check for the distribution of the probiotic in gut conditions using a powdered dye.

Principle:

Distribution of the probiotic is important to combat limitations of survivability due to presence of some other organisms or enzymatic activity etc. Hence a powdered dye which is insoluble in water can be used to simulate an open drug.

Materials required:

SAUL, powdered water insoluble dye, Semi solid medium, 1M $NaHCO_3$, Guar gum, distilled water.

Procedure:

Tank components

Tank 1	Semi-solid medium (Full stomach), Empty stomach – Guar gum in distilled water to make the viscosity between 10^3 Pa-s and as low as 10^{-2} Pa-s (To simulate mucous) [6].
Dry inlet	Indigo dye powder (Insoluble in water)
Buffers	1M $NaHCO_3$

Simulation of the stress conditions of the drug distribution in the small intestine:

- Add the powdered dye initially into the device just before the dialysis tube opening.
- Add 200mL semi-solid medium at at 0.129 RPM (2.5ml/min) (0.261 RPM (4.0mL/min), 0.412 RPM (8mL/min), 0.618 (12mL/min), 0.773 RPM (15mL/min), 1.031 RPM (20mL/min) [11] (or mucous substitute) and maintain pH at 7.4 using the buffer.
- Repeat the experiment with different flow rates, temperatures and viscosities of the semi-solid medium and mucous substitute.
- Plot a graph of parameter vs time.

Note: The above steps are performed with SAUL in horizontal configuration (6 hours).

Observation:

- Distribution of the drug can be mapped.

Results:

- A distribution profile is obtained.

[6] Lai S. K. et al., 2009 [12] Rao S. P. et al., 2017

C. Bacteria

i. Extent of stress tolerance of bacteria

Aim: To check for stress tolerance of bacteria in gut conditions.

Principle:

Bacteria is subjected to different stress conditions in the human gut. Bile salts and sudden variations in pH are one of the most important contributors of viability of the probiotic bacteria. Survivability of different strains varies in these environments.

Materials required:

SAUL connected to tanks, *Escherichia coli Nissle 1917* in nutrient broth (in the appropriate CFU count, Semi solid medium, bile salts, 1M $NaHCO_3$, Guar gum, distilled water.

Procedure:

Tank components

Tank 1	Semi-solid medium (Full stomach), Empty stomach – Guar gum in distilled water to make the viscosity between 10^3 Pa-s and as low as 10^{-2} Pa-s (To simulate mucous) [6] .
Tank 2	*Escherichia coli Nissle 1917* in nutrient broth (in the appropriate CFU count)
Tank 3	1M $NaHCO_3$
Tank 4	4% Bile salt solution
Outlet	Mixture to be analyzed

Simulation of the stress conditions of the bacteria in the small intestine:

- Pump the semi-solid medium into the reactor at 0.380 RPM (2.5ml/min), 0.609 RPM (4.0mL/min), 1.218 RPM (8mL/min), 1.827 (12mL/min), 2.283 RPM (15mL/min), 3.044 RPM (20mL/min) [11].
- Introduce the *Escherichia coli Nissle 1917* in nutrient broth (in the appropriate CFU count) into the reactor.
- Neutralize the contents of the device to pH 6 by adding 1M $NaHCO_3$ at a rate of 4.5 ml/min to simulate the passing of food from the stomach to the duodenum.

Note: The time taken for neutralization can vary depending on the buffering capacity of the food.

- Add 4% of bile salts solution to make the concentration in the device to 0.4% at rate of 4 ml/min during a period of 10 min.
- Dilute the bioreactor at D=0.4/h using the dilution medium to simulate the absorption of bile acids and food components in the jejunum and ileum. Maintain pH at 6.5 during the process.
- Gradually increase the pH to 9 and then reduce to 7.4 and maintain the flow at a constant flow rate through the reactor (recycle stream).
- Repeat the experiment with different flow rates, temperatures and viscosities of the semi-solid medium and mucous substitute.
- Plot a graph of OD vs time with readings taken every 10 minutes.

Note: The above steps are performed with SAUL in horizontal configuration (6 hours).

Observation:

- Tolerance of the bacteria in the presence and absence of bile can be checked.

Results:

- Viability of the bacteria can be determined.

[6] Lai S. K. et al., 2009 [12] Rao S. P. et al., 2017

ii. Dynamic Analysis of conversion efficiency

a. Methylmercury

Aim: To check for conversion efficiency of probiotic in a dynamic system.

Principle:

The small intestine is a complex dynamic environment with a variety of physiological and mechanical stresses in play. These properties play an important role in determining the viability of the probiotic drug components. In vivo studies are too complicated and expensive to be used for the initial screening process of studying the chassis to be used, checking the effectiveness of the genes in a variable dynamic environment and/or in optimization of the capsule for appropriate delivery. Current lab scale methodologies involve an oversimplified static system which would not replicate most properties of its target site environment. They do not subject the organisms to continuously varying stresses which the organisms experience in vivo.

Materials required:

SAUL connected to tanks, Transformed *Escherichia coli Nissle 1917* in nutrient broth (in the appropriate CFU count, Semi solid medium, bile salts, 1M $NaHCO_3$, Guar gum, distilled water, methylmercury.

Circuits:

Constitutive Promoter – RBS – **MerR - PmerT promoter - (**RBS – **MerT** – RBS – **MerP** – RBS – **MerE** – RBS **- MerC)*-** RBS – **MerA** – RBS – **MerB** – RBS – **GFP - Double Terminator**

Control:

Wild type Escherichia coli Nissle 1917

Procedure:

Tank components

Tank 1	Semi-solid medium (Full stomach), Empty stomach – Guar gum in distilled water to make the viscosity between 10^3 Pa-s and as low as 10^{-2} Pa-s (To simulate mucous) [6] .
Tank 2	*Escherichia coli Nissle 1917* in nutrient broth (in the appropriate CFU count)
Tank 3	1M $NaHCO_3$
Tank 4	4% Bile salt solution
Outlet	Mixture to be analyzed

Simulation of the stress conditions of the bacteria in the small intestine:

- Add 0.3µM of methylmercury chloride into the semi-solid medium and mix until uniform.
- Pump the metal induced semi-solid medium into the reactor at 0.129 RPM (2.5ml/min) (0.261 RPM (4.0mL/min), 0.412 RPM (8mL/min), 0.618 (12mL/min), 0.773 RPM (15mL/min), 1.031 RPM (20mL/min) [11].
- Neutralize the contents of the device to pH 6 by adding 1M $NaHCO_3$ at a rate of 4.5 ml/min to simulate the passing of food from the stomach to the duodenum.
- Pump the *Escherichia coli Nissle 1917* in nutrient broth (in the appropriate CFU count) into the reactor.

Note: The time taken for neutralization can vary depending on the buffering capacity of the food.

- Add 4% of bile salts solution to make the concentration in the device to 0.4% at rate of 4 ml/min during a period of 10 min.
- Dilute the bioreactor at D=0.4/h using the dilution medium to simulate the absorption of bile acids and food components in the jejunum and ileum. Maintain pH at 6.5 during the process.
- Gradually increase the pH to 9 and then reduce to 7.4 and maintain the flow at a constant flow rate through the reactor (recycle stream).
- Measure the amount of methylmercury in the outlet using gas chromatography at every stage of induction or every 10 minutes.
- Repeat the experiment with different flow rates, methylmercury concentrations and viscosities of the semi-solid medium and mucous substitute.
- Plot a graph of mercury concentration vs time with readings taken every 10 minutes.

Note: The above steps are performed with SAUL in horizontal configuration (6 hours).

Observation:

- Methylmercury conversion efficiency can be mapped.

Results:

- The bacteria are extremely efficient in converting methylmercury to elemental mercury.

[6] Lai S. K. et al., 2009 [12] Rao S. P. et al., 2017

b. Methylmercury and oxidative stress conditions

Aim: To check for overall efficiency of the probiotic in a dynamic system.

Principle:

The small intestine is a complex dynamic environment with a variety of physiological and mechanical stresses in play. These properties play an important role in determining the viability of the probiotic drug components. In vivo studies are too complicated and expensive to be used for the initial screening process of studying the chassis to be used, checking the effectiveness of the genes in a variable dynamic environment and/or in optimization of the capsule for appropriate delivery. Current lab scale methodologies involve an oversimplified static system which would not replicate most properties of its target site environment. They do not subject the organisms to continuously varying stresses which the organisms experience in vivo.

Materials required:

SAUL connected to tanks, Transformed *Escherichia coli Nissle 1917* in nutrient broth (in the appropriate CFU count, Semi solid medium, bile salts, 1M $NaHCO_3$, Guar gum, distilled water, methylmercury and paraquat.

Circuits:

Final transformed probiotic bacteria with BioBrick 2 containing GFP

Control:

Wild type Escherichia coli Nissle 1917

Procedure:

Tank components

Tank 1	Semi-solid medium (Full stomach), Empty stomach – Guar gum in distilled water to make the viscosity between 10^3 Pa-s and as low as 10^{-2} Pa-s (To simulate mucous) [6].
Tank 2	*Escherichia coli Nissle 1917* in nutrient broth (in the appropriate CFU count)
Tank 3	1M $NaHCO_3$
Tank 4	4% Bile salt solution
Outlet	Mixture to be analyzed

Simulation of the stress conditions of the bacteria in the small intestine:

- Add 0.3µM of methylmercury chloride into the semi-solid medium and mix until uniform.
- Pump the metal induced semi-solid medium into the reactor at 0.380 RPM (2.5ml/min), 0.609 RPM (4.0mL/min), 1.218 RPM (8mL/min), 1.827 (12mL/min), 2.283 RPM (15mL/min), 3.044 RPM (20mL/min) [11].
- Neutralize the contents of the device to pH 6 by adding 1M $NaHCO_3$ at (volume/min) at a rate of 4.5 ml min−1 to simulate the passing of food from the stomach to the duodenum.
- Pump the *Escherichia coli Nissle 1917* in nutrient broth (in the appropriate CFU count) into the reactor.
- Intermittently (According to the results of the previous experiments, we can determine the time interval) add paraquat to induce oxidative stress conditions in the reactor.

Note: The time taken for neutralization can vary depending on the buffering capacity of the food.

- Add 4% of bile salts solution to make the concentration in the device to 0.4% at rate of (Volume) 4 ml min−1 during a period of 10 min.
- Dilute the bioreactor at D=0.4/h using the dilution medium to simulate the absorption of bile acids and food components in the jejunum and ileum. Maintain pH at 6.5 during the process.
- Gradually increase the pH to 9 and then reduce to 7.4 and maintain the flow at a constant flow rate through the reactor (recycle stream).
- Measure the amount of methylmercury in the outlet using gas chromatography at every stage of induction or every 10 minutes.
- Every half hour pump 40µM of paraquat into the reactor
- Repeat the experiment with different flow rates, methylmercury concentrations and viscosities of the semi-solid medium and mucous substitute.
- Plot a graph of mercury concentration vs time and GFP vs time with readings taken every 10 minutes.

Note: The above steps are performed with SAUL in horizontal configuration (6 hours).

Observation:

- Methylmercury conversion efficiency can be mapped.
- Inflammatory response can be mapped.

Results:

- The bacteria are extremely efficient in converting methylmercury to elemental mercury.
- The inflammatory response of the bacteria is mapped.

[6] Lai S. K. et al., 2009 [12] Rao S. P. et al., 2017

4. Glossary

Term	Definition	Ref
Absorption	Absorption is a condition in which something takes in another substance.	Link
Accumulation	A mass or quantity of something that has gradually gathered or been acquired	Link
Agarose	Agarose is a polysaccharide, generally extracted from certain red seaweed. It is a linear polymer made up of the repeating unit of agarobiose, which is a disaccharide made up of D-galactose and 3,6-anhydro-L-galactopyranose.	Link
Amplification of DNA	PCR amplification is the selective amplification of DNA or RNA targets using the polymerase chain reaction.	Link
Anatomy	Anatomy is the identification and description of the structures of living things. It is a branch of biology and medicine.	Link
Annealing	When the temperature is lowered to enable the DNA primers to attach to the template DNA.	Link
Antibiotics	Antibiotics are medicines that help stop infections caused by bacteria. They do this by killing the bacteria or by keeping them from copying themselves or reproducing. The word antibiotic means “against life”.	Link
Apical chamber	Apical (anatomy), an anatomical term of location for features located opposite the base of an organism or structure.	Link
Autoclave	An autoclave is a machine that uses steam under pressure to kill harmful bacteria, viruses, fungi, and spores on items that are placed inside a pressure vessel.	Link
Basolateral chamber	Located in or on the base and one or more sides	Link

Bile	Bile (from latin bilis), or gall, is a dark-green-to-yellowish-brown fluid produced by the liver of most vertebrates that aids the digestion of lipids in the small intestine. In humans, bile is produced continuously by the liver (liver bile) and stored and concentrated in the gallbladder.	Link
BioBrick	BioBrick parts are DNA sequences that follow a specific restriction enzyme assembly standard.	Link
Bioreactor	A bioreactor refers to any manufactured device or system that supports a biologically active environment.	Link
Bolus	In digestion, a bolus is a ball-like mixture of food and saliva that forms in the mouth during the process of chewing.	Link
Boundary conditions	The set of conditions specified for the behavior of the solution to a set of differential equations at the boundary of its domain.	Link
Cell suspension	A cell suspension or suspension culture is a type of cell culture in which single cells or small aggregates of cells are allowed to function and multiply in an agitated growth medium, thus forming a suspension.	Link
CFU	A colony forming unit, or CFU, is a unit commonly used to estimate the concentration of microorganisms in a test sample.	Link
Chassis	In synthetic biology, a chassis refers to an organism that houses and supports genetic components by providing the resources that allow them to function.	Link
Compressive force	The force acting on a body that causes reduction in dimensions of the body along the direction in which it is applied.	Link
Configuration	An arrangement of parts or elements in a particular form, figure, or combination.	Link

Contamination	Contamination is the presence of a constituent, impurity, or some other undesirable element that spoils, corrupts, infects, makes unfit, or makes inferior a material, physical body, natural environment, workplace, etc.	Link
Contraction	Muscle contraction is the activation of tension-generating sites within muscle fibers.	Link
Control DNA	Control DNAs are powerful tools in the investigation of genomic DNA methylation and epigenomic research. They can be utilized in many protocols where they can serve as comparisons to sample DNA.	Link
DC motor	A DC motor is any of a class of rotary electrical motors that converts direct current electrical energy into mechanical energy.	Link
Deep-groove ball bearings	Deep groove, or single row radial, ball bearings are the most widely used bearings. They utilize an uninterrupted raceway that makes them optimal for radial loads. This design permits precision tolerance, even at high-speed operation.	Link
Deformation	Deformation refers to the change in size or shape of an object.	Link
Deionized water	Deionized water (DI water, DIW or de-ionized water), often synonymous with demineralized water / DM water, is water that has had almost all of its mineral ions removed, such as cations like sodium, calcium, iron, and copper, and anions such as chloride and sulfate.	Link
Density	Density is a measure of mass per volume. The average density of an object equals its total mass divided by its total volume.	Link
Digestion	Digestion is the breakdown of large insoluble food molecules into small water-soluble food molecules so that they can be absorbed into the watery blood plasma.	Link

Dilution	Dilution is the process of reducing the concentration of a solute in solution, usually simply by mixing with more solvent.	Link
Dimension	Measurement in a particular direction	Link
Double digestion of DNA	Digesting a DNA substrate with two restriction endonucleases simultaneously (double digestion) is a common timesaving procedure.	Link
Electrodes	An electrode is a solid electric conductor that carries electric current into non-metallic solids, or liquids, or gases, or plasmas, or vacuums. Electrodes are typically good electric conductors, but they need not be metals. Cathode and Anode.	Link
Electrophoresis	Electrophoresis is the motion of dispersed particles relative to a fluid under the influence of a spatially uniform electric field. Electrophoresis of positively charged particles is sometimes called cataphoresis, while electrophoresis of negatively charged particles is sometimes called anaphoresis.	Link
Enzymes	Enzymes are biological molecules (typically proteins) that significantly speed up the rate of virtually all of the chemical reactions that take place within cells.	Link
Equimolar	Equimolar is having the same amount of moles.	Link
Flow pattern	The flow pattern is the system for movement of raw materials from the beginning to the end where it is converted into finished or final product.	Link
Flow rate	Volumetric flow rate is the volume of fluid which passes per unit time; usually it is represented by the symbol Q.	Link
Frequency	Frequency is the number of occurrences of a repeating event per unit of time.	Link

Friction	Friction is a force between two surfaces that are sliding, or trying to slide, across each other.	Link
Gas Stripping	Stripping is a physical separation process where one or more components are removed from a liquid stream by a vapor stream.	Link
Gastric juices	Gastric acid, gastric juice, or stomach acid is a digestive fluid formed within the stomach lining.	Link
Gastrointestinal tract	The gastrointestinal tract is the tract from the mouth to the anus which includes all the organs of the digestive system in humans and other animals.	Link
Growth factors	A growth factor is a naturally occurring substance capable of stimulating cell proliferation, wound healing, and occasionally cellular differentiation.	Link
Hydrophobicity	Hydrophobicity is the physical property of a molecule that is seemingly repelled from a mass of water.	Link
Incubate	Incubation period is the time needed for any particular process of development to take place.	Link
Inoculation	The act of introducing microorganism or suspension of microorganisms (e.g. bacteria) into a culture medium.	Link
Laminar flow	Laminar flow is a flow regime characterized by high momentum diffusion and low momentum convection.	Link
Ligate	In molecular biology, ligation is the joining of two nucleic acid fragments through the action of an enzyme.	Link
Load	Load is a term frequently used in engineering to mean the force exerted on a surface or body.	Link
Mesh	The finite element mesh is a grid made up of small elements, used to subdivide the CAD model into smaller domains over which a set of equations are solved.	Link

Molecular cloning	Molecular cloning is a set of techniques used to insert recombinant DNA from a prokaryotic or eukaryotic source into a replicating vehicle such as plasmids or viral vectors.	Link
Molecular sieve	A molecular sieve is a material with pores (very small holes) of uniform size. These pore diameters are similar in size to small molecules, and thus large molecules cannot enter or be adsorbed, while smaller molecules can.	Link
Motility	Gastrointestinal (GI) motility refers to the movement of food from the mouth through the pharynx (throat), esophagus, stomach, small and large intestines and out of the body.	Link
Muscle	Muscle is a soft tissue found in most animals. Muscle cells contain protein filaments of actin and myosin that slide past one another, producing a contraction that changes both the length and the shape of the cell. Muscles function to produce force and motion.	Link
Nucleases	A nuclease (also archaically known as nucleodepolymerase or polynucleotidase) is an enzyme capable of cleaving the phosphodiester bonds between nucleotides of nucleic acids. Nucleases variously effect single and double stranded breaks in their target molecules.	Link
Nucleotides	Nucleotides are organic molecules consisting of a nucleoside and a phosphate. They serve as monomeric units of the nucleic acid polymers deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), both of which are essential biomolecules within all life-forms on Earth.	Link
Osmotic balance	Osmoregulation is the process of maintenance of salt and water balance (osmotic balance) across membranes within the body's fluids, which are composed of water, plus electrolytes and non-electrolytes.	Link

Palindromic sequences	A palindromic sequence is a nucleic acid sequence in a double-stranded DNA or RNA molecule wherein reading in a certain direction (e.g. 5' to 3') on one strand matches the sequence reading in the same direction (e.g. 5' to 3') on the complementary strand.	Link
Peristalsis	Peristalsis is the progressive wave of contraction and relaxation of a tubular muscular system, especially the alimentary canal, by which the contents are forced through the system.	Link
pH	pH is a quantitative measure of the acidity or basicity of aqueous or other liquid solutions. A solution with a pH less than 7 is considered acidic; a solution with a pH greater than 7 is considered basic, or alkaline.	Link
Physiological	Physiological condition or, more often "physiological conditions" is a term used in biology, biochemistry, and medicine. It refers to conditions of the external or internal milieu that may occur in nature for that organism or cell system, in contrast to artificial laboratory conditions.	Link
Poisson's Ratio	Poisson's ratio is defined as the ratio of the change in the width per unit width of a material, to the change in its length per unit length, as a result of strain.	Link
Polymerization	Polymerization is a process of reacting monomer molecules together in a chemical reaction to form polymer chains or three-dimensional networks.	Link
Positive Control	A positive control is a group in an experiment that receives a treatment with a known result, and therefore should show a particular change during the experiment. It is used to control for unknown variables during the experiment and to give the scientist something to compare with the test group.	Link

Primers	A primer is a short, single-stranded DNA sequence used in the polymerase chain reaction (PCR) technique. In the PCR method, a pair of primers is used to hybridize with the sample DNA and define the region of the DNA that will be amplified. Primers are also referred to as oligonucleotides.	Link
Printing filament	Printing filament is the raw material used for 3D printing. Filaments used in 3D printing are thermoplastics that melt rather than burn when heated, can be shaped and molded, and solidify when cooled.	Link
Probiotic	Live microorganisms that are similar to beneficial microorganisms found in the human gut that are taken as dietary supplements or found in foods.	Link
Pump	A pump is a device that moves fluids by mechanical action, typically converted from electrical energy into Hydraulic energy.	Link
Radially symmetrical	Symmetry about a central axis.	Link
Reagents	A reagent is a substance or compound added to a system to cause a chemical reaction, or to test if a reaction occurs.	Link
Regenerated cellulose	Regenerated cellulose is a class of materials manufactured by the conversion of natural cellulose to a soluble cellulosic derivative and subsequent regeneration, typically forming either a fiber or a film.	Link
Relaxation	That phase of a muscle twitch during which a muscle returns to a resting position.	Link
Restriction sites	Restriction sites, or restriction recognition sites, are located on a DNA molecule containing specific sequences of nucleotides, which are recognized by restriction enzymes.	Link
Screening processes	Detection and isolation of a microorganism from a natural environment like soil containing large number of microbial population is called as screening.	Link

Segmentation	Segmentation is the process of mixing the food contents in the digestive tract to create a more uniform mixture throughout.	Link
Semi-permeable	A material or membrane that allows certain substances to pass through it but not others, especially allowing the passage of a solvent but not of certain solutes.	Link
Semi-solid	Having the qualities of both a solid and a liquid, highly viscous.	Link
Shaft	A shaft is a rotating machine element, usually circular in cross section, which is used to transmit power from one part to another.	Link
Standard deviation	The standard deviation is a statistic that measures the dispersion of a dataset relative to its mean and is calculated as the square root of the variance.	Link
Static	Pertaining to or characterized by a fixed or stationary condition.	Link
Sterility	Sterility is the state of being free from biological contaminants.	Link
Sterilization	Sterilization is a process that destroys or eliminates all forms of microbial life and is carried out by physical or chemical methods.	Link
Stock solution	A stock solution is a concentrated solution that will be diluted to some lower concentration for actual use.	Link
Stresses	The internal restoring forces per unit area that develop in a body subjected to an external load.	Link
Thermocycling reaction	Thermocycling is the use of a thermocycler to amplify DNA segments.	Link
Torque	Torque is the tendency of a force to rotate the body to which it is applied.	Link

Unidirectional	Moving or operating in one direction only.	Link
Viability	Ability of a microbe to survive under given conditions.	Link
Viscosity	Viscosity is the measure of a fluid's resistance to flow.	Link
Worm gear	A worm gear is a gear consisting of a shaft with a spiral thread that engages with and drives a toothed wheel.	Link
Young's Modulus	Young's modulus is a measure of the ability of a material to withstand changes in length when under lengthwise tension or compression. It is the ratio of longitudinal stress to strain within elastic limit.	Link

5. References

Definitions and physiology:

https://medlineplus.gov/ency/article/003883.htm#:~:text=The%20normal%20volume%20of%20the,%2Fhr)%20in%20some%20cases.

https://www.ncbi.nlm.nih.gov/books/NBK535425/

https://en.wikipedia.org/wiki/Peristalsis

https://www.innerbody.com/image_dige02/dige21.html#continued

https://en.wikipedia.org/wiki/Basal_electrical_rhythm#:~:text=The%20frequency%20of%20contraction%20differs,to%20a%20phenomenon%20called%20mass

http://scienceline.ucsb.edu/getkey.php?key=5055

https://www.healthline.com/human-body-maps/jejunum#:~:text=The%20jejunum%20is%20one%20of,intestine%20to%20the%20large%20intestine.

https://core.ac.uk/download/pdf/8780062.pdf

Preparation of media:

- https://www.k-state.edu/hermanlab/protocols/AntibioticUsage.html#:~:text=To%20obtain%20this%20in%20100ml%20of%20LB%2C%20add%20100ul%20stock,LB%2C%20add%20100ul%20stock%20solution.
- http://mcb.berkeley.edu/labs/krantz/protocols/agar_plates.pdf

PCR amplification:

- https://www.neb.com/protocols/0001/01/01/pcr-protocol-m0530

Restriction digestion:

- https://international.neb.com/protocols/2018/07/30/restriction-digest-protocol

Agarose gel electrophoresis

- https://www.mun.ca/biology/scarr/Gel_Electrophoresis.html#:~:text=Gel%20electrophoresis%20separates%20DNA%20fragments,medium%20(an%20agarose%20gel).&text=The%20rate%20of%20migration%20is,an%20intercalating%20dye%2C%20ethidium%20bromide.

Gibson assembly:

- https://2019.igem.org/wiki/images/8/89/T--TU_Eindhoven--ProtocolGibson.pdf

Competent cell preparation:

- https://www.iitg.ac.in/biotech/MTechLabProtocols/Expt-4%20(Preparation%20of%20Competent-cells).pdf
- https://byjus.com/biology/competent-cells/
- http://2012.igem.org/Team:Penn/Nissle#:~:text=Production%20of%20Chemically%20Competent%20Nissle,to%20OD600%20~0.25%2D0.3.

Bacterial transformation

- https://www.addgene.org/protocols/bacterial-transformation/

Papers:

[1] Adusumali R-B, Reifferscheid M, Weber H, Roeder T, Sixta H, Gindl W (2006) Mechanicalproperties of regenerated cellulose fibres for composites. Macromol Symp 244:119–125 https://doi.org/10.1002/masy.200651211

[2] Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K. (1998). Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract. Journal of applied microbiology, 84(5), 759–768. https://doi.org/10.1046/j.1365-2672.1998.00407.x

[3] Fine, K. D., Santa Ana, C. A., Porter, J. L., & Fordtran, J. S. (1995). Effect of changing intestinal flow rate on a measurement of intestinal permeability. Gastroenterology, 108(4), 983–989. https://doi.org/10.1016/0016-5085(95)90193-0

[4] Gregersen, H., & Kassab, G. (1996). Biomechanics of the gastrointestinal tract. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society, 8(4), 277–297. https://doi.org/10.1111/j.1365-2982.1996.tb00267.x

[5] Jacobsen, C. N., Rosenfeldt Nielsen, V., Hayford, A. E., Møller, P. L., Michaelsen, K. F., Paerregaard, A., Sandström, B., Tvede, M., & Jakobsen, M. (1999). Screening of probiotic activities of forty-seven strains of Lactobacillus spp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans. Applied and environmental microbiology, 65(11), 4949–4956. https://doi.org/10.1128/AEM.65.11.4949-4956.1999

[6] Lai, S. K., Wang, Y. Y., Wirtz, D., & Hanes, J. (2009). Micro- and macrorheology of mucus. Advanced drug delivery reviews, 61(2), 86–100. https://doi.org/10.1016/j.addr.2008.09.012

[7] Lankaputhra, W.E.V., Shah, N.P., 1995. Survival of Lactobacillus acidophilus and Bifidobacterium spp. in the presence of acid and bile salts. Cultured Dairy Products Journal 30, 2 – 7

[8] Li, J., Bi, X., Zhang, K. *et al.* Experimental study on the effects of shear stress on viscoelastic properties of the intestines. *Sci. China Technol. Sci.* **62,** 1028–1034 (2019). https://doi.org/10.1007/s11431-018-9428-4

[9] Mainville, I., Arcand, Y., & Farnworth, E. R. (2005). A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics. International journal of food microbiology, 99(3), 287–296. https://doi.org/10.1016/j.ijfoodmicro.2004.08.020

[10] Nakamura, K., Wada, M., Kuga, S., & Okano, T. (2004). Poisson's Ratio of Cellulose Iα and cellulose II. *Journal of Polymer Science, Part B: Polymer Physics*, *42*(7), 1206–1211. https://doi.org/10.1002/polb.10771

[11] Prasad, J., Gill, H., Smart, J., Gopal, P.K., 1998. Selection and characterisation of Lactobacillus and Bifidobacterium strains for use as probiotics. International Dairy Journal 8, 993 – 1002. https://doi.org/10.1016/s0958-6946(99)00024-2

[12] Rao, P.S.; Reddy, G.B.; Reddy, V.D. Design and development of advanced rotary peristaltic pump. Int. J. Mech. Eng. Technol. 2017, 8, 695–703.