iGEM MIT_MAHE
Product Design and Development Handbook

Acknowledgements

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1. Introduction

The manufacturing of a final product design of a drug/probiotic involves three stages.

Upstream processing in development of a probiotic involves

- Selection and modification of a strain according to requirements.
- Selection and optimization of media used to grow the strain.
- Selection of a capsule formulation and testing.
- Selection of excipients.

During this phase the active pharmaceutical ingredient (API) (in our case the modified probiotic) is determined. This includes stages up to clinical trials and industrial production.

Bioprocessing involves growing of the bacteria in a bioreactor.

Downstream processing involves

- Separation: Separation of the API from other components.
- Purification: Purification of the API to ensure there are no impurities or contaminants.
- Manufacturing of the required capsule: For the drug delivery process.
- Recycling and waste management:

Then the finished product is sent for **packaging** which involves covering our product with a packing material (cold form film in our case) to ensure the integrity of the product.

Our process for the manufacturing is divided into 5 parts,

- Powder manufacturing process.
- Empty capsule manufacturing process.
- Capsule filling process.
- Packaging process.
- Recycling, waste management, and safety process.

For safety consideration: Refer to iGEM MIT_MAHE safety handbook

Note: Further experimentation will be required where some experiments are designed in iGEM MIT_MAHE product development experiments handbook.

2. Powder design and development

A. Upstream processing/ Pre-production:

Strain development:

The strain development process involves the preparation and testing of recombinant plasmids in the *Escherichia coli* Nissle 1917 with the insert described in the design part of the project. Initially, in order to ensure safety, we had proposed a design where the antibiotic resistance gene was removed from the bacteria so that any unnecessary resistances would not be imparted to the natural gut biota.

However, **Dr. Keyur Raval** said that the presence of antibiotic-resistance genes would create a plasmid conservation pressure during production which would ensure that plasmid loss would not occur as well as prevent contamination of media. Hence, each plasmid will have a different antibiotic resistance gene in the final strain developed for production.

However, inclusion of such genes in the final strain design poses the threat of imparting unnecessary antibiotic resistance to the natural gut microbiome. When we voiced our concerns about this, he suggested the usage of CRISPR Cas 9 technology to integrate our design to the genome of the bacteria. However, we decided against it as the long term effects of this technology is not known. In addition, *Escherichia coli* Nissle 1917 does not have the ability to undergo conjugation and thus cannot participate in Horizontal Gene Transfer which made us more confident about the traditional method.

The recombinant plasmids must be prepared in lab with the insert described in the design part of the project. The plasmid and bacteria will be tested using multiple experiments as designed in iGEM MIT_MAHE Composite Bio-Brick 1 handbook and iGEM MIT_MAHE Composite Bio-Brick 2 handbook. This recombinant bacterium will be then used for the manufacturing process; hence this prepared bacterial strain will be cryopreserved till the time need for its use.

Metabolic stoichiometry

Empirical equation:

Carbon source: Glycerol

Basis: 1 mole of Glycerol

$$C_3H_8O_3 + aO_2 + bNH_3 \rightarrow cCH_\alpha O_\beta N_\gamma + dCO_2 + eH_2O$$

Where

- a is number of moles of oxygen
- b is number of moles of ammonia utilized (any nitrogen source always gets taken up as ammonia)
- c is the number of moles of biomass
- d is the number of moles of carbon-di-oxide
- e is the number of moles of water

Metabolic stoichiometry:

Basis: 1 mole of glycerol

Biomass equation for *Escherichia coli* Nissle 1917 → $CH_{1.77}O_{0.49}N_{0.24}$

$$C_3H_8O_3 + aO_2 + bNH_3 \rightarrow cCH_{1.77}O_{0.49}N_{0.24} + dCO_2 + eH_2O$$

Biomass = 12 x 1 + 1.77 x 1 + 0.49 x 16 + 0.24 x 14 = 24.97g

Number of electrons in glycerol: 3 x 4 + 8 x 1 + 3 x (-2) = 14 e^-

Each electron pertains to roughly 3.12 → π g of biomass

43.96g of biomass per 1 mole (approx.) of glycerol

$$C_3H_8O_3 + aO_2 + bNH_3 \rightarrow 1.76CH_{1.77}O_{0.49}N_{0.24} + dCO_2 + eH_2O$$

N: $b = c \times \gamma$ $b = 1.76 \times 0.24$ $b = 0.4224$	**C:** $1 \times 3 = c \times 1 + d \times 1$ $1 \times 3 = 1.76 \times 1 + d \times 1$ $d = 1.24$
H: $1 \times 8 + b \times 3 = c \times \alpha + e \times 2$ $1 \times 8 + 0.4224 \times 3 = 1.76 \times 1.77 + e \times 2$ $e = 3.076$	**O:** $1 \times 3 + 2 \times a = c \times \beta + d \times 2 + e \times 1$ $1 \times 3 + 2 \times a = 1.76 \times 0.49 + 1.24 \times 2 + 3.076 \times 1$ $a = 1.7092$

Biomass formula for *E Coli*: Link (Accessed 14th October)

Final metabolic stoichiometry:

$$C_3H_8O_3 + \ 1.7092\ O_2 + 0.4224\ NH_3 \rightarrow \ 1.76\ CH_{1.77}O_{0.49}N_{0.24} + 1.24\ CO_2 + 3.076\ H_2O$$

Respiratory co-efficient: $\frac{Coeff.of\ CO_2}{Coeff.of\ O_2} = \frac{1.24}{1.7092} = 0.72676$

Theoretical oxygen demand: $\frac{1.7092\ \ mol\ of\ oxygen}{1\ mol\ glycerol} \times \frac{32\ g\ of\ oxygen}{92\ g\ of\ glycerol} = \frac{0.5945\ g}{g}$

Media formulation:

Based on the stoichiometry, media can be formulated.

The largest cost in any bioprocess is incurred due to raw materials. Different regions of the world would have different readily available and cheap nitrogen sources. To ensure least expensive production, we have curated a list of different states of India and the cheapest nitrogen source available. Hence the media formulation would vary from state to state depending on availability.

Nitrogen source	State
Soybean Liquor	Rajasthan
	Madhya Pradesh
	Maharashtra
	Andhra Pradesh
	Chhattisgarh
	Gujarat
	Karnataka
Groundnut Meal	Gujarat
	Rajasthan
	Andhra Pradesh
	Tamil Nadu
	Maharashtra
	Karnataka
	Madhya Pradesh
	Telangana
	West Bengal
	Uttar Pradesh
	Orissa
Corn Steep Liquor	Bihar
	Uttar Pradesh
	Rajasthan
	Madhya Pradesh
	Andhra Pradesh

Yeast extract	Andhra Pradesh
	Telangana
	Kerala
	Karnataka
	Sikkim
	Haryana
	Himachal Pradesh
Cottonseed Flour	Gujarat
	Maharashtra
	Andhra Pradesh
	Madhya Pradesh
	Punjab
	Haryana

For example, if we consider our set up to be in Karnataka, Corn steep liquor is the most economical nitrogen source (around Rs.30 per Kg) (through literature). Studies also show that it is more efficient when corn steep liquor is used along with peanut meal/Soybean meal. But the effect of these combinations on *E. coli* would have to be checked (this adds up to another Rs.25/kg). The combinations and concentrations can be finalized during media optimization.

The nitrogen content of each source can be calculated using the experiments mentioned in iGEM MIT_MAHE Product development experiments handbook.

Depending on the nitrogen content in the source and the stoichiometry, the amount of nitrogen source to be added can be calculated.

Initially, the carbon source proposed was **glucose** and **tryptone**.

"Components which are a potential food resource for humans and animals must not be used for microbes."
~Dr Keyur Raval

Hence, **glycerol** was chosen - it is also cheap and readily available across all regions of India.

B. Bioprocess:

Through surveys we have found that the rate of consumption of fishes (primary source of methylmercury poisoning) in each state is different (yearly).

State	**Total house-holds**	**Number of households reported Consumption per 1000 households {(R+U)/2}**	**No. of members per household**	**Total households that consume fish**	**Total persons consuming fish (A)**	**Per Capita Consumption (kg) (R+U)**	**Rural people who might not consume pill (50% rural pop. that consumes fish)**
Andhra Pradesh	12712077	143	3.9	1817827	7089525	1.56	2156603
Arunachal Pradesh	354801	551	3.9	195495	762430	7.38	181613
Assam	6639484	777	4.7	5158879	24246731	8.64	6272320
Bihar	19277676	262	5.4	5050751	27274055	2.58	7781433
Chhattisgarh	5321916	160	(NA) 4.8	851506	4087228	1.56	1270873
Goa	347272	823	4.2	285804	1200380	19.44	305564
Gujarat	12859508	69	4.7	887306	4170338	0.6	1042584
Haryana	4783294	10	5.3	47832	253509	0.12	63377
Himachal Pradesh	1492304	12	4.6	17907	82372	0.24	20593
J&K	2200181	16	5.7	35202	200651	0.12	50162
Jharkhand	6108913	171	5.4	1044624	5640969	1.74	1410242
K'taka	13281586	98	4.6	1301595	5987337	1.68	1496834
Madhya Pradesh	14525361	65	5	944148	4720740	0.6	1180185
Maharashtra	23909432	156	4.7	3729871	17530393	1.56	4382598
Manipur	514078	738	5	379389	1896945	6.18	474236
Meghalaya	581742	666	5.1	387440	1975944	5.52	493986
Mizoram	228584	214	4.8	48916	234796	2.28	58699
Nagaland	439667	326	4.5	143331	644989	4.62	161247
Odisha	9327604	534	4.5	4980940	22414230	5.52	5603557
Punjab	5335257	4	5.2	21341	110973	0	27743
Rajasthan	12463352	8	5.5	99706	548383	0.06	137095
Sikkim	135683	44	4.5	5970	26865	0.36	6716
TN+ Telangana	30614486	241	3.5	7378091	25823318	3.3	6455829
Tripura	854399	948	4.3	809970	3482871	15.9	870717

State	Total house-holds	Number of households reported Consumption per 1000 households {(R+U)/2}	No. of members per household	Total households that consume fish	Total persons consuming fish (A)	Per Capita Consumption (kg) (R+U)	Rural people who might not consume pill (50% rural pop. that consumes fish)
Uttarakhand	2017258	49	5	98845	494225	0.78	123556
UP	35054796	76	5.7	2664164	15185734	0.66	3796433
WB	20283581	811	4.5	16449984	74024928	11.04	18506232
A and N island	79287	847.5	(NA)4.8	67156	322348	16.98	80587
Chandigarh	211090	3	5	633	3165	0.06	791
D&N Haveli	71302	158	(NA)4.8	11265	54072	1.32	13518
Daman and Diu	50731	519	(NA)4.8	26329	126379	4.38	31594
Delhi	3497466	54	(NA)4.8	188863	906542	0.42	226635
Lakshadweep	13431	869	(NA)4.8	11671	56020	36.72	14005
Puducherry	259990	702	(NA)4.8	182512	876057	7.38	219014

At first, we considered designing a bioreactor having a volume of 10000L which we changed to a more need based approach, taking in his advice. Thus, based on the amount of fish consumed per region, the amount of pills to be manufactured per region was estimated and listed (refer to handbook). Therefore, to reduce **costs** and **wastage**, **three bioreactors** of **different sizes** were designed based on **low, moderate** and **high** requirements.

Bioreactor design:

Dr. Vytla Ramachandra Murty advised us to use a stirred batch fermentor as the bioreactor.

We have taken **3 sizes**

1. 1000L = 1 m^3= 10^9 mm^3
2. 10000L = 10m^3= 10^{10} mm^3
3. 100000L= 100m^3= 10^{11} mm^3

Parameters:

Height/diameter ratio is H:D = between 3 and 4

For (1) D_1 = x, H_1 = 3.714x, V_1 = 10^9 mm^3

$$\pi \frac{D^2}{4} H = \text{Volume}$$

$$\pi\ 3.714\ \frac{x^3}{4} = 10^9$$

For (2) $D_2 = x$, $H_2 = 3.419x$, $V_2 = 10^{10}$ mm^3

$$\pi \frac{D^2}{4} H = \text{Volume}$$

$$\pi\ 3.419\ \frac{x^3}{4} = 10^{10}$$

For (3) $D_3 = x$, $H_3 = 3.545x$, $V_3 = 10^{11}$ mm^3

$$\pi \frac{D^2}{4} H = \text{Volume}$$

$$\pi\ 3.545\ \frac{x^3}{4} = 10^{11}$$

Diameters:

$D_1 = x_1 = 700$ mm (approx.) for 1000L

$D_2 = x_2 = 1550$ mm (approx.) for 10000L

$D_3 = x_3 = 3300$ mm (approx.) for 100000L

Heights:

$H_1 = 2600$mm for 1000L

$H_2 = 5300$m for 10000L

$H_3 = 117000$mm for 100000L

Number of impellers = 2 for all of the reactors

Type of impeller → 6 blade curved bladed impeller → **SCaBa 6SRGT**

Position of impellers (from base):

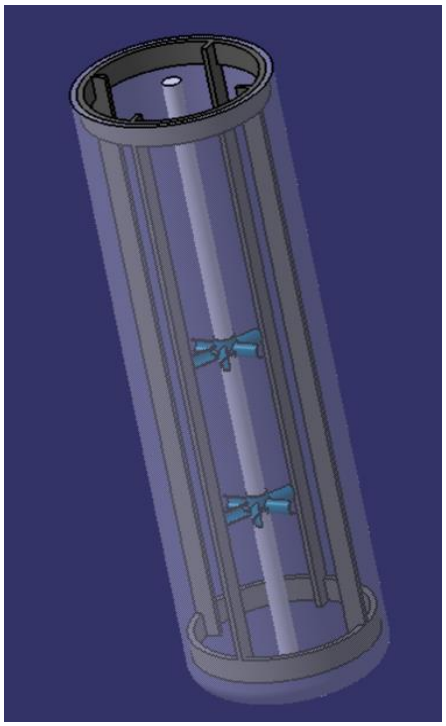
1000L	**10000L**	**100000L**
700mm	1400mm	2100mm
1550mm	3100mm	4650mm

Baffle width = $\frac{D}{10}$ for all

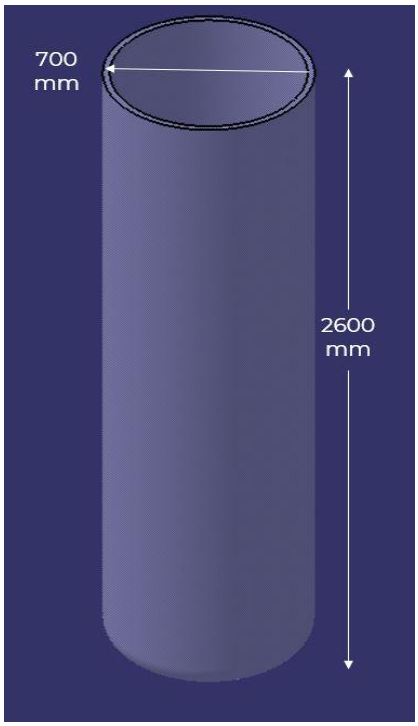
Final measurements:

Volume (L)	Diameter (mm)	Height (mm)	Number of impellers	Position of impellers (mm)		Baffle width (mm)	Number of baffles
1000	**700**	**2600**	**2**	**700**	**1400**	**70**	**4**
10000	**1550**	**5300**	**2**	**1550**	**3100**	**155**	**4**
100000	**3300**	**11700**	**2**	**3300**	**6600**	**330**	**4**

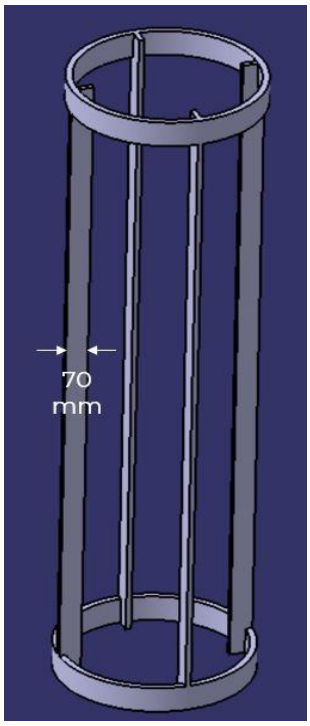
Bioreactor diagram:



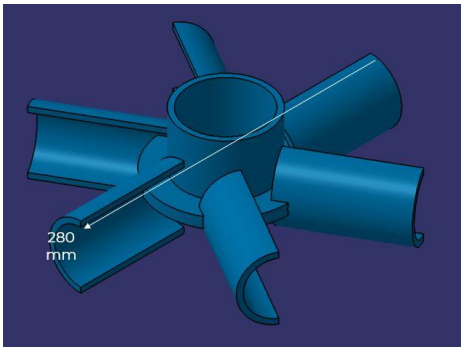
Bioreactor assembly



Tank



Baffles



Impeller

The estimated amount of powder and pills to be manufactured per month:

High fish consumption

State	People who consume fish and might consume the pill	Dosage (per month	Total pills per month	Pills for kids (45% of the total)	Pills for adults (55% of the total)	Weight of active component in kids pill in g (16mg per pill)	weight of active component in adult pills in g (30mg per pill)
Tripura	2612153	6	15672918	7052813.1	8620104.9	112845.0096	258603.147
Lakshadweep	42015	6	252090	113440.5	138649.5	1815.048	4159.485
A and N island	241761	6	1450566	652754.7	797811.3	10444.0752	23934.339
Goa	900285	6	5401710	2430769.5	2970940.5	38892.312	89128.215
WB	55518696	6	333112176	149900479.2	183211696.8	2398407.667	5496350.904
Assam	18185048	6	109110288	49099629.6	60010658.4	785594.0736	1800319.752
TOTAL	**77499958**		**464999748**	**209249886.6**	**255749861.4**	**3347998.186**	**7672495.842**

Moderate fish consumption

State	People who consume fish and might consume the pill	Dosage (per month	Total pills per month	Pills for kids (45% of the total)	Pills for adults (55% of the total)	Weight of active component in kids pill in g (16mg per pill)	weight of active component in adult pills in g (30mg per pill)
Arunachal Pradesh	571822	2	1143644	514639.8	629004.2	8234.2368	18870.126
Odisha	16810672	2	33621344	15129604.8	18491739.2	242073.6768	554752.176
Meghalaya	1481958	2	2963916	1333762.2	1630153.8	21340.1952	48904.614
Manipur	1422709	2	2845418	1280438.1	1564979.9	20487.0096	46949.397
Daman and Diu	94784	2	189568	85305.6	104262.4	1364.8896	3127.872
Puducherry	657043	2	1314086	591338.7	722747.3	9461.4192	21682.419
TOTAL	**21038988**		**42077976**	**18935089.2**	**23142886.8**	**302961.4272**	**694286.604**

Low fish consumption

State	People who consume fish and might consume the pill	Dosage (per month	Total pills per month	Pills for kids (45% of the total)	Pills for adults (55% of the total)	Weight of active component in kids pill in g (16mg per pill)	weight of active component in adult pills in g (30mg per pill)
Andhra Pradesh	5317144	1	5317144	2392714.8	2924429.2	38283.4368	87732.876
Bihar	20455541	1	20455541	9204993.45	11250547.55	147279.8952	337516.4265
Chhattisgarh	3065421	1	3065421	1379439.45	1685981.55	22071.0312	50579.4465
Gujarat	3127753	1	3127753	1407488.85	1720264.15	22519.8216	51607.9245
Haryana	190132	1	190132	85559.4	104572.6	1368.9504	3137.178
Himachal Pradesh	61779	1	61779	27800.55	33978.45	444.8088	1019.3535
J&K	150488	1	150488	67719.6	82768.4	1083.5136	2483.052
Jharkhand	4230727	1	4230727	1903827.15	2326899.85	30461.2344	69806.9955
K'taka	4490503	1	4490503	2020726.35	2469776.65	32331.6216	74093.2995
Madhya Pradesh	3540555	1	3540555	1593249.75	1947305.25	25491.996	58419.1575
Maharashtra	13147795	1	13147795	5916507.75	7231287.25	94664.124	216938.6175
Mizoram	176097	1	176097	79243.65	96853.35	1267.8984	2905.6005
Nagaland	483742	1	483742	217683.9	266058.1	3482.9424	7981.743
Punjab	83230	1	83230	37453.5	45776.5	599.256	1373.295
Rajasthan	411287	1	411287	185079.15	226207.85	2961.2664	6786.2355
Sikkim	20149	1	20149	9067.05	11081.95	145.0728	332.4585
TN+ Telangana	19367488	1	19367488	8715369.6	10652118.4	139445.9136	319563.552
Uttarakhand	370669	1	370669	166801.05	203867.95	2668.8168	6116.0385
UP	11389300	1	11389300	5125185	6264115	82002.96	187923.45
Chandigarh	2374	1	2374	1068.3	1305.7	17.0928	39.171
D&N Haveli	40554	1	40554	18249.3	22304.7	291.9888	669.141
Delhi	679906	1	679906	305957.7	373948.3	4895.3232	11218.449
total	**90802634**		**90802634**	**40861185.3**	**49941448.7**	**653778.9648**	**1498243.461**
TOTAL						**4304738.578**	**9865025.907**

C. Downstream processing/ Production:

Ms Archana Mahadev Rao gave us a checklist to follow whilst designing our process. Given a product and a desired annual production rate bioprocess design endeavours to answer several questions

- What are the required amounts of raw materials and utilities?
- What is the required size of process equipment and supporting utilities?
- What is the manufacturing cost?
- What is the optimum batch size?
- How long does a single batch take?
- How much product can be generated per year?
- What is the demand for resources in the course of a batch?
- What is the amount of resources consumed?
- Bottlenecks?
- Environmental impact?

The production process for the probiotic has considered this checklist and involves several steps:

Media preparation:

In a sterile tank the media formulation (after optimization tests) is added along with adequate amount of water.

Sterilization:

Ultra-high temperature sterilization will be employed for this process.

Ultra-high temperature processing (UHT), is a sterilization technique which sterilizes media by heating it above 135°C (275 °F) which is required to kill bacterial endospores to ensure complete sterilization.

Advantages:

- **High quality:** Process time is shorter due to higher temperature having minimal come-up and cool-down time leading to a higher quality product.
- **Longer shelf life:** A shelf life of longer than 6 months without refrigeration can be expected.
- **Packaging size:** Conditions are independent of the size of the container.

Fermentation in the bioreactor:

In biochemistry, fermentation is narrowly defined as the extraction of energy from carbohydrates in the absence of oxygen.

The bioreactor as designed above will be used after adequate testing.

- The required quantity of sterilized media will be pumped into the bioreactor.
- The strain developed in the upstream process will be revived and used to make inoculum.
- The strain will be mixed with the same media formulation as the one used in the bioreactor for preparing the inoculum (frozen seed stock method).

Centrifugation:

Centrifugation is the method of separating molecules having different densities. This is done by spinning them in solution around an axis at high speed inside a centrifuge rotor.

- The fermented product produced will be subjected to centrifugation for removing excess media or other metabolites and impurities.
- Thus, a liquid having only the recombinant probiotic bacterium will be obtained. This will not have any other substances like side products or raw materials.

The centrifuge used will be a density gradient centrifuge.

Filtration:

Filtration is an operation that separates solids or fluids from a mixture with a filter medium that has a complex structure through which only fluids can pass.

- The centrifuge product will be subjected to filtration.
- This will ensure that a liquid of higher probiotic concentration will be obtained by removing excess water (i.e maintain minimal amount of water)

Note: The filtration and centrifuge parameters used will be selected such that minimum stresses on the bacteria is ensured and cells will remain intact after the processes.

Cryo and lyo protectant addition

Cryoprotectants inhibit the rate of ice growth by increasing the solution viscosity as well as keep the amorphous structure of ice in proximity of the cell and are thus used to protect cells from injury during freezing process. Lyoprotectants stabilize the lipid bilayer structure of the cellular membrane in the absence of water and are used to protect cells during the freeze-drying process.

- The filtrate is pumped into a sterile tank and the desired cryoprotectants and Lyoprotectants are mixed into it.
- For our needs initially we took,
    - Cryoprotectant- Sucrose and sodium phosphates in water
    - Lyoprotectant- Sucrose

However, Dr. Keyur Raval advised us to use components which are cannot be used as food for humans and animals.

- Hence, we have changed to
    - Cryoprotectant- Glycerol and sodium phosphates in water
    - Lyoprotectant- Glycerol

All of the components are considered extremely safe for human consumption. However, more experiments are required to determine the same.

Pelletizing:

Pelletizing is the process of compressing or molding materials into the shape of a pellet.

- Cryoprotected concentrate will be allowed to drip through calibrated holes into a liquid nitrogen bath. These pellets are typically spheres of 4-5mm in diameter.
- Harvesting is done at the bottom.
- Freezing by formation of cell pellets can be followed by freeze-drying (lyophilization) resulting in a dried end product.

Freeze drying:

Freeze drying is a process which removes water or moisture and is typically used for preservation of perishable items. It works by freezing the material, reducing the pressure and then adding heat to allow the frozen water to sublimate.

- The frozen pellets are transferred onto trays which will placed on the shelves in the freeze-drying chamber, and then vacuum is applied, these shelves are progressively heated once vacuum is established.
- The applied vacuum range typically varied between 100-1000 mTorr with the shelves' temperature between −40 and +40 °C.
- The time duration of the process varies as a function of the strain, its formulation, and the freeze-drying cycle, usually takes a few days to be completed.
- Freeze drying maintains the probiotic cells at a low temperature, thus limiting the damage to the cell's structure and metabolites.

Milling:

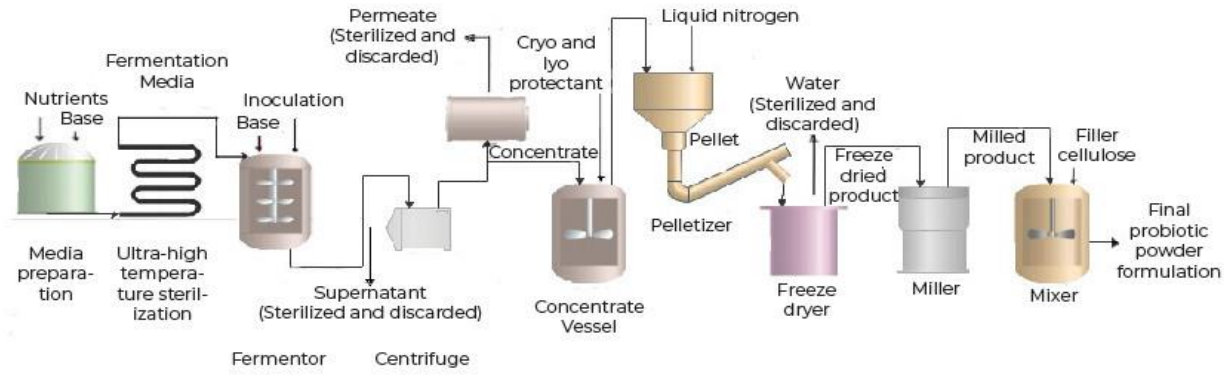
Milling is a powerful unit operation which aids in the control of particle size.

- Milling results in a powder with defined particle size and density.

A quality control system will be present just after the milling process to ensure no significant genetic drift has occurred due to several factors such as metabolic burden.

The probiotic powder will then be mixed with the appropriate amounts of fillers. It will be ensured that the fillers will not contain any known allergens so that almost everyone can use the product.

The filler which would be used is cellulose. This would also help people achieve their daily limit of dietary fibres.



Powder manufacturing flowchart

3. Product design

The final product will be a probiotic enteric 2-piece capsule filled with recombinant bacteria along with other fillers as mentioned. There are two variants of this;

- Child variant containing 8 billion CFU per capsule.
- Adult variant containing 15 billion CFU per capsule.

Note: The numbers are based on the safe and required number of CFU for a probiotic product (Link).

The capsule will be filled with a certain weight of the active pharmaceutical ingredient (API) as produced above (recombinant probiotic powder) such that each capsule variant attains the specific CFU limit set earlier. This weight of the API will be decided based on the CFU/mg value that is attained.

To calculate the CFU/mg

- Calculate the fermentation time using the number of generations and doubling time values. In our estimation:
  Generation time – 20
  Doubling time – 25 minutes
  Fermentation time = Generation time x Doubling time = 20 x 25 = 500 minutes = 8 hours 20 minutes.
- Collect the sample after the fermentation time and measure the amount of CFU/mL as given by the experiment in iGEM MIT_MAHE product development experiments handbook.
- Multiply the bioreactor volume and CFU/mL value to obtain the total amount of biomass in the reactor. In our estimation:
  Reactor volume – 1000L, 10000L and 100000L
  Total Biomass = Reactor volume x CFU/mL = CFU/volume of the reactor
- Divide the total biomass by density of the mixture in the reactor to get CFU/mass of the reactor and then convert to CFU/mg.

Based on the amount of API, the best suitable capsule size will be selected from the standard capsule size chart given below. The filler will be mixed with the API so that the total product being filled into the capsule is considerably close to the typical fill weight as per the capsule size.

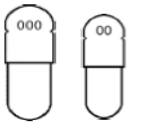
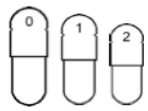
Representative (approximate) Capsule Sizes	Capsule Size	Typical Fill Weights (mg) Actual Fill Weights may vary and depend on powder characteristics			Volume Theoretical	Locked Length		External Diameter	Cut Length	Single Wall Thickness	Weight (Avg. of 100)
		Powder Density			(ml)	+/- 0.76 (mm)	Tolerance / Component	(mm)	+/- 0.51 (mm)	+/- 0.03 (mm)	+/-10% (mg)
		0.45 (Light)	0.70 (Typical)	1.00 (Heavy)							
	000	615	960	1370	1.37	26.14	Cap	9.91	12.95	0.112	163
							Body	9.55	22.20	0.110	
	00	430	665	950	0.95	23.30	Cap	8.53	11.74	0.109	118
							Body	8.18	20.22	0.107	
	0	305	475	680	0.68	21.70	Cap	7.65	10.72	0.107	96
							Body	7.34	18.44	0.104	
	1	225	350	500	0.50	19.40	Cap	6.91	9.78	0.104	76
							Body	6.63	16.61	0.102	
	2	165	260	370	0.37	18.00	Cap	6.35	8.94	0.102	61
							Body	6.07	15.27	0.099	
	3	135	210	300	0.30	15.90	Cap	5.82	8.08	0.092	48
							Body	5.56	13.59	0.890	
	4	95	145	210	0.21	14.30	Cap	5.31	7.21	0.096	38
							Body	5.05	12.19	0.091	
	5	60	90	130	0.13	11.10	Cap	4.91	6.20	0.089	28
							Body	4.68	9.32	0.086	

Image source

Formulation of the capsule:

The probiotic will be filled in a two-piece enteric capsule, with the following formulation [6] :

Formulation	HPMC (% w/w)	Gellan gum (% w/w)	Sodium alginate (% w/w)	NaCl (% w/w)
1	17	0.2	2	0.2

Role of the components:

- **HPMC**: Hypromellose or hydroxypropyl methylcellulose is a semisynthetic, inert, viscoelastic polymer which is the base material for our capsule. It protects the contents from degradation or product changes, which means insulating against temperature fluctuations, moisture exposure, etc. Thus, it helps in preserving the integrity of the product.
- **Gellan gum**: It is a water-soluble anionic polysaccharide artificially produced by the non-pathogenic bacterium *Sphingomonas elodea* using lactose. Its ability to form gels on addition of calcium ions makes it a suitable gelling agent. Its advantages include biodegradability, non-toxicity, rapid gelation in the presence of cations, etc [5].
- **Sodium alginate**: Sodium alginate is a cell wall component of marine brown algae. It is used in variety of products including capsules due to its properties such viscosity enhancement, stabilizer, matrixing agent, encapsulation polymer, bioadhesive which makes it versatile [4]. It is also acid insoluble providing an acid resistant coating to probiotic capsules [6] .
- **NaCl**: It is sodium chloride which plays a role by helping in the gelling of gellan gum.

[4] Sachan N. K. et al., 2009 [5] Osmalek et al., 2014 [6] Smith, A. M. et al., 2010

The capsule was tested and the following results were obtained

- approached the 2-h intact requirement with a rupture time of 75 min and 85 min, respectively.
- passed the enteric test and remained intact for the 2 h timeframe.
- loaded with 100 mg diclofenac were also subjected to an enteric test using the USP I apparatus (baskets). Both capsule formulations passed the enteric test retaining their shape.

Formulations	G'(Pa) at 25°C	G''(Pa) at 25°C	G'(Pa) at 60°C	G''(Pa) at 60°C	Temperature @ G'Max (°C)
1	464.1	274.8	3.9	2.0	43.7

Where,

G' is storage modulus

G'' is loss modulus

4. Process design

A. Empty capsule manufacturing process:

Dipping method of capsule manufacturing process will be used which involves 7 steps:

Preparation of the dipping solution:

To ensure that the cap and body will attain different colours the process will occur in two batches.

- The required quantities of gellan gum, sodium alginate and NaCl would be dissolved in deionized water at temperature > 80°C in a sterile tank of required volume.
- One batch would be added with the desired artificial colour and the other batch with the other desired colour.
- Then the required quantity of HPMC (based on the table above and the amount of deionized water to be used) is dispersed into this polymer mixture of both the batches.
- The solution is then stirred for 2hours until all the material is fully hydrated and a homogenous dispersion is evident. This is the required dipping solution.

Dip coating process:

Capsules shells are manufactured under strict climatic conditions by dipping the standardized steel pins arranged in rows on a metal bars into the dipping solution.

- The steel pins corresponding to the body will be arranged separately in one bar and the pins corresponding to the cap in the other.
- Each of these bars will be dipped into their respective batch of dipping solution, which would be maintained at temperature around 58°C, to attain a thickness of around 280 micrometers.

The thickness of the coating is directly proportional to the viscosity of the coating liquid and viscosity of the liquid is inversely proportional to the temperature of the liquid.

Hence, thickness of the coating is inversely proportional to the temperature of the coating liquid. This dependency is shown by the graph below for the formulation we selected for our capsule.

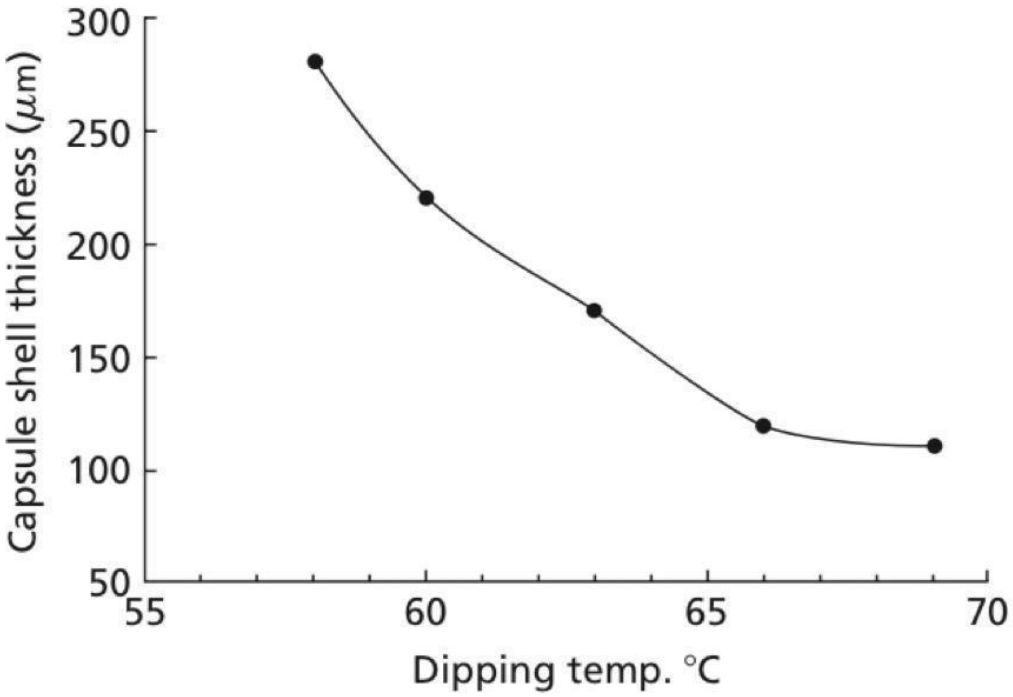


Figure 2 Dipping temperature vs capsule thickness for formulation 5 (17% HPMC 0.2% gellan gum, 2% sodium alginate, 0.2% NaCl). Results represent mean ± SD, $n = 3$.

Image source

Rotating of the dip-coated pins:

After the pins are coated with the dipping solution, the bar containing these pins will be rotated multiple times to ensure an even coating ensuring an evenly thick capsule.

Drying:

Once the rotating process is completed the pins are left to dry under a blast of cool air, till the required moisture content is achieved. Several capsules with different amounts of water (by different times subjected to drying) will be tested for various stresses like acid, strength etc.

Stripping and trimming:

After the capsules are dried, they are stripped from the pins and are trimmed to a proper length.

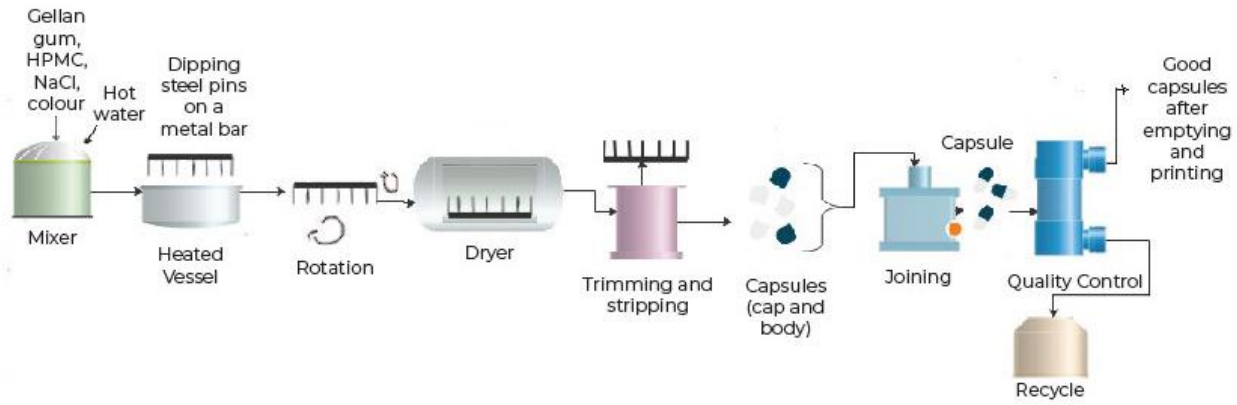
Joining of the trimmed capsule shell:

As the name of this process suggest the 2 pieces of the capsule shell thus produced from the above process is joined. The pieces that are not good enough is discarded and will be recycled.

Printing:

The following information will be printed on to the surface of the capsule shell using food safe colour.

- Name of the products i.e. the brand name.
- The total amount of powder that will be filled i.e. both the active substance and the fillers



Empty capsule manufacturing flowchart

B. Capsule filling:

An automatic capsule filling machine can separate, fill, and bolt capsules sequentially. Such a machine can extraordinarily improve the efficiency of production and decrease labour costs. This machine includes 7 internal steps which are described as below,

Capsules rectification

Since most of the newly manufactured capsules are joined - they must be rectified before further processes.

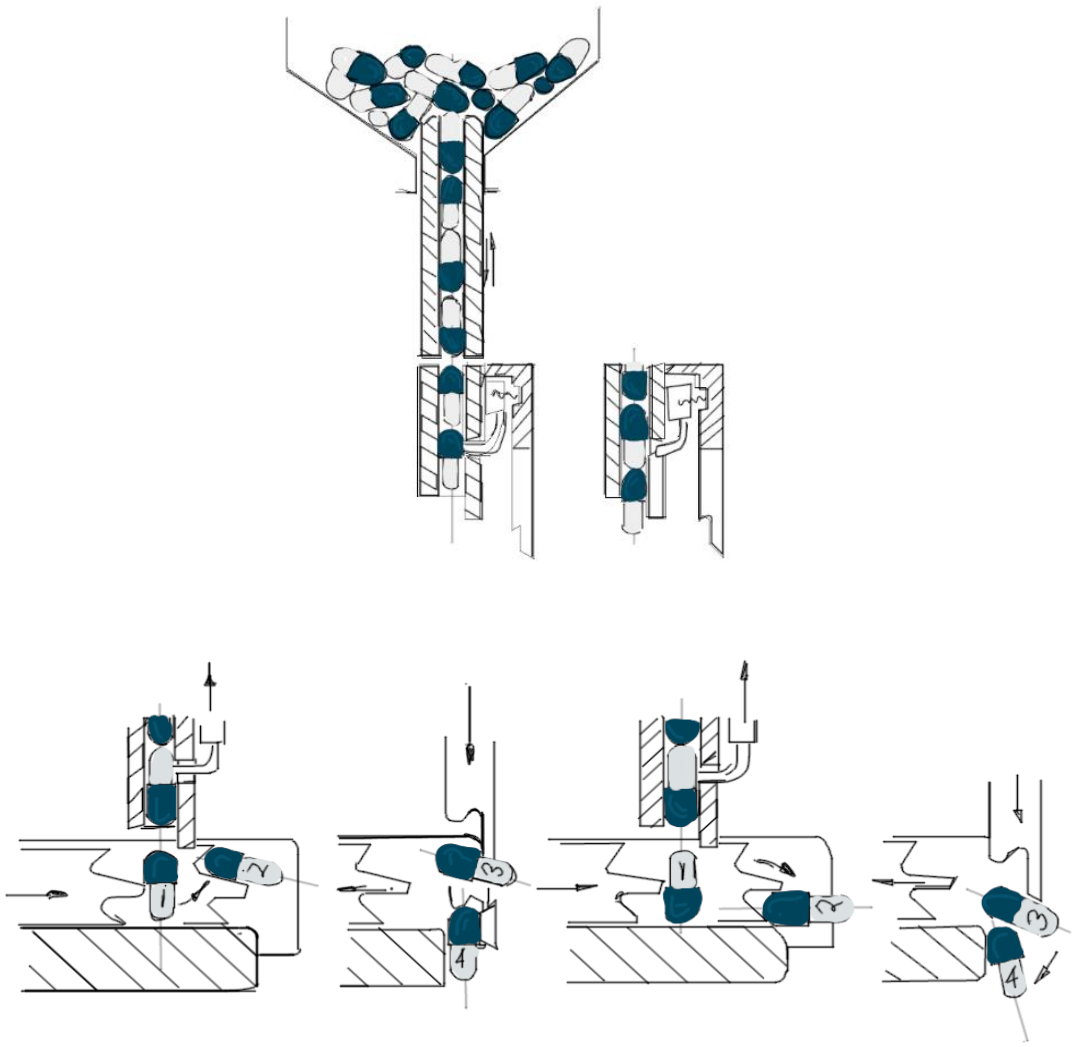
- Capsules are continuously pumped onto the delivery plate of the capsule with a multitude of circular channels inside.
- These channels are ideal for the capsule to traverse through, and the lower portion of each channel is filled with spring bits.
- During operation, the capsule delivery plate reciprocates up and down and allows the capsules to penetrate the holes and to be bound to the spring portion.
- Then the capsule delivery plate goes down to allow the spring portion to free the capsules, and then the capsules fall.
- Capsules might require more rectification as either they may fall body-end downward or the cap-end downward.
- The capsule falls in front of the horizontal fork.

Owing to its unique shape, the horizontal fork only works on the middle portion of the capsules whose diameter is smaller.

Owing to the different centre of gravity, the capsule will be pushed down with the body of the capsule ahead, irrespective of its original conformation.

The position of the capsule can be changed by the vertical fork again such that all the capsules enter the segment with the body of the capsule down. This marks the end of the capsule rectification.

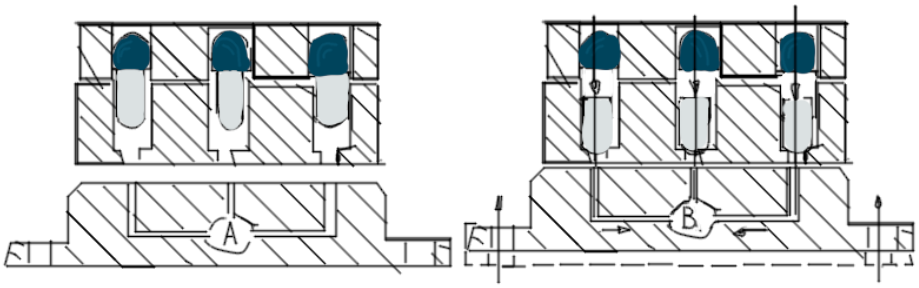
Generally, the automated capsule filling system has various versions available based on the capsule tray hole - for e.g. 9 holes for 1200C, 18 holes for 2000C, and 24 holes for 3500C. It indicates how many capsules the system will fill at a time and thus impact the overall filling speed. Most automatic capsule fillers will fill capsules of various sizes by adjusting the design.



Separation of capsule caps from bodies

- The capsule tray is moved to the capsule separator, and the vacuum divider is raised to render its upper surface similar to the lower surface of the lower portion without a gap.
- The capsule would be isolated by vacuum due to the difference between the width of the caps and bodies of the capsule.
- Capsule caps will be attached to the upper portion of the capsule tray, and the interior of the capsule will go down.

After separation, the upper and lower parts of the capsule tray would be divided and moved to their respective stations.



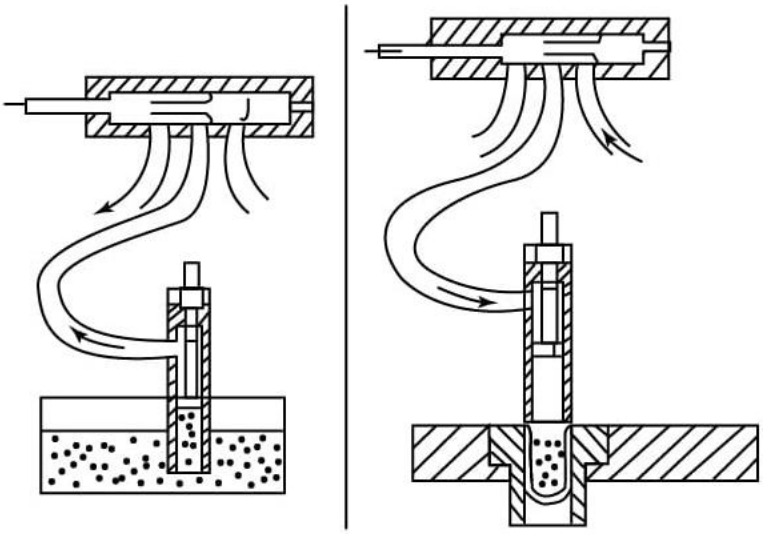
Filling medications

The body of the capsule will be delivered to the filling station for filling the medication. There are many ways to fill medication, mostly tamping filling, intermittent dosing filling, dosing cylinder filling and vacuum filling are used. But we will plan on using the vacuum filling because of reasons mentioned below,

Vacuum filling

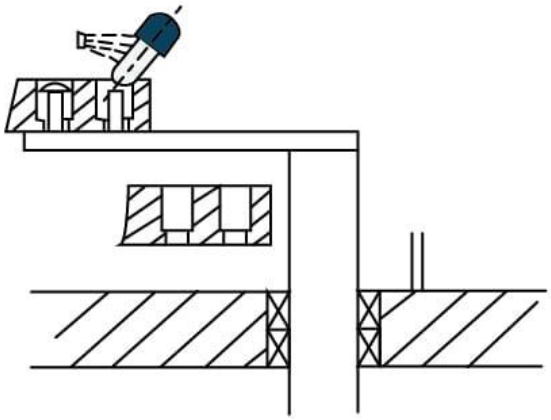
Vacuum filling is used to vacuum the drugs into the dosing tank and then to use compressed air to blast drugs into the interior of the capsule.

It is relevant to all forms of drug, no mechanical moving parts, and the damage to the drug is the least equivalent to any other form of filling.



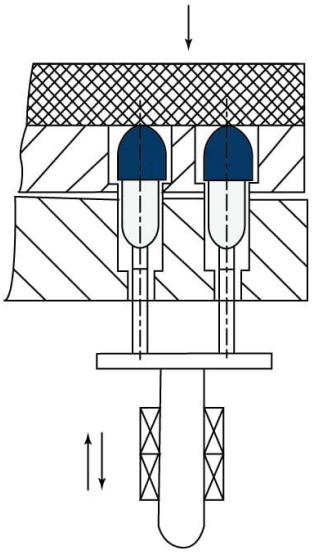
Wasted capsules rejection

If any empty capsules are not separated for whatever reason. There will be pins corresponding to the capsules. If the cap of the capsule is not removed from the body of the capsule, the pin will force the capsule out of the mold, and the capsule will be blown into the capsule collecting bags. The capsule caps and the bodies that are separated will be moved to the next process.



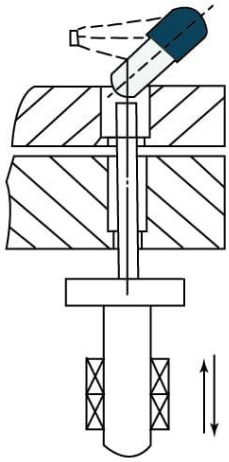
Capsule Locking

Upper and lower capsule trays rotate at the same time and at this moment the upper and lower capsule tray axis lines converge. The baffle plate above the capsule tray and the pin below the tray continue to rotate to render the capsule closed.



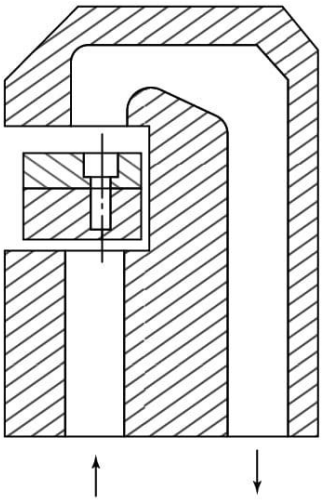
Capsule Ejection

The capsule ejection mechanism is like the capsule rejection mechanism, the pin forces the locked capsule out of the capsule tray, and then the compressed air throws the ejected capsules into the outlet.



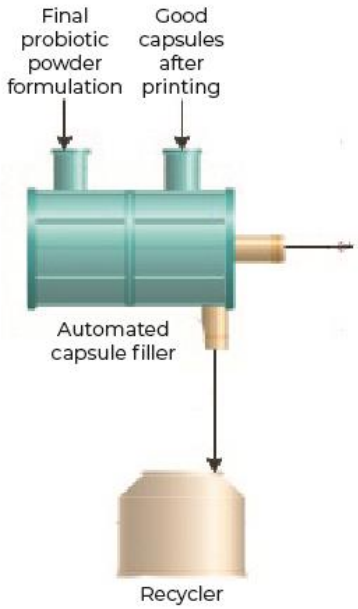
Cleaning

The capsule tray will travel back to the first capsule rectification station after the whole filling phase has been completed. But the powder or discarded capsules might remain in the capsule tray which needs to be washed off. Applying compressed air from the bottom can help blow out the powder and wasted capsules to the dust collection device.



Each step takes a short time, and the rational circular layout enables all steps to continue at the same time. As a result, the automated capsule filling system can fill 12k-450k capsules per hour at the fastest speed and is the best option for large-volume capsule development.

This machine will be presented as a single unit further on as seen below,



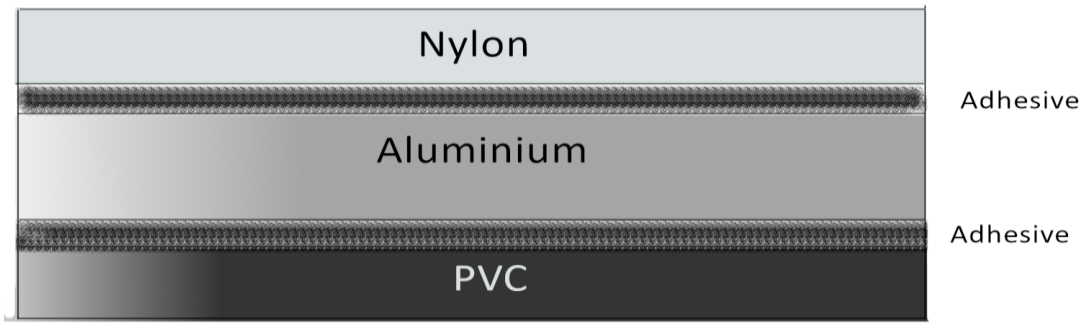
Automated capsule filler flowchart

5. Packaging

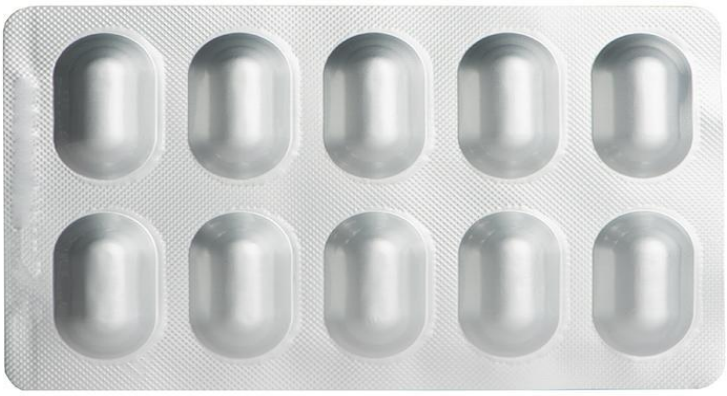
The packaging material is an important aspect of the product development process as it helps protect the integrity of the product.

The packing material must be moisture and light proof as both these elements can cause changes in the nature of the product and contributes to reducing its shelf life or making it less efficient.

Hence, we propose on using cold form film as the forming film to form a blister pack. Cold form film is a multi-layered film made using PVC, aluminium foil, and polyamide like nylon each of them layered on upon the other and fixed in place by using an adhesive layer in between each of them. The PVC side is on the inside in contact with the product.



Cold form film layers



Cold form film blister pack

Image source

The lidding material will be aluminium push through foil with a paper on top.

- The paper and the aluminium foil will be attached using an adhesive layer with the aluminium being the inner side.
- A layer of heat seal coating will to added to the region where the lidding material will be attached to the blisters to form a good seal.
- The paper, adhesive sheet, aluminium foil, and heat seal coating together make up the lidding material.

For blister packs this heat seal coating would be the most important component as an ineffective coating will lead to an ineffective seal resulting in an aluminium lidding material which would be of no use.

Also, a good heat seal coating and heat seal will also increase the aesthetic look of the blister packing. The paper outer layer on the lidding material form a good material for printing proposes.

Layers of lidding material

Some **disadvantages** of cold film form blister are,

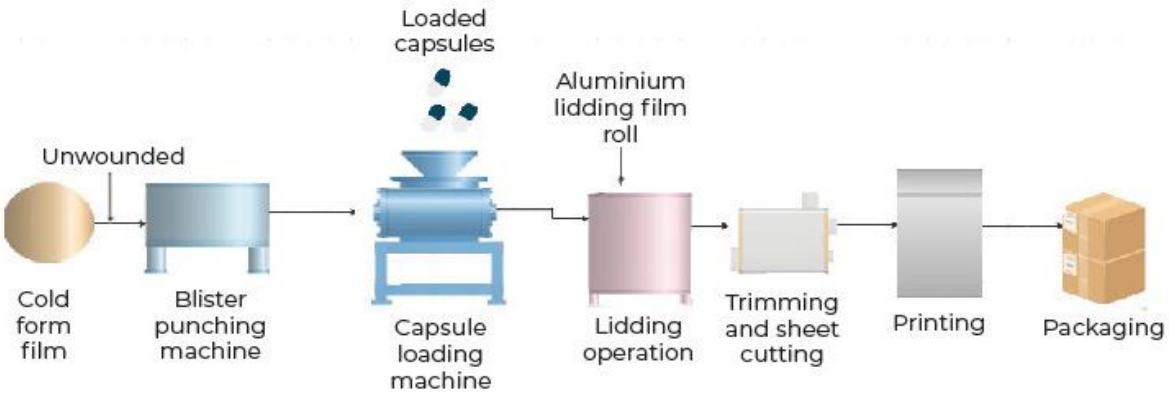
- Higher cost per unit of packing material
- Higher time required for the process as production process is slower
- The packing is opaque hence the blister inspection system much more difficult and time consuming and hence expensive.
- This type of packing produces larger size as aluminium can't be moulded to 90^{o} so the blister can't have 90^{o} angles as the cold form film has aluminium in them.

However, the **advantages** make up for them,

- The aluminium used provides an almost complete barrier to moisture, temperature, oxygen etc. hence increasing the effective shelf life of the product inside it.
- Since we have a probiotic product this will also help in prevent the probiotic to grow as there would not be oxygen for them to propagate and hence helping in preventing any possible genetic drift.

The operations involved in forming this cold form film blister packs (cold forming) are;

- Installing the cold form film.
- Forming cavities (i.e. Blisters) into the sheets of the cold form film using the punch moulds such that it produces two rows of blisters side by side.
- Loading the products into these newly formed blisters.
- Placing the lidding materials (same dimensions as that of the pack).
- Heating seal the packs.
- Trimming and cutting the continuous sheets into sets of 10 in each.
- Packing 10 of these blister pack into cardboard boxes.



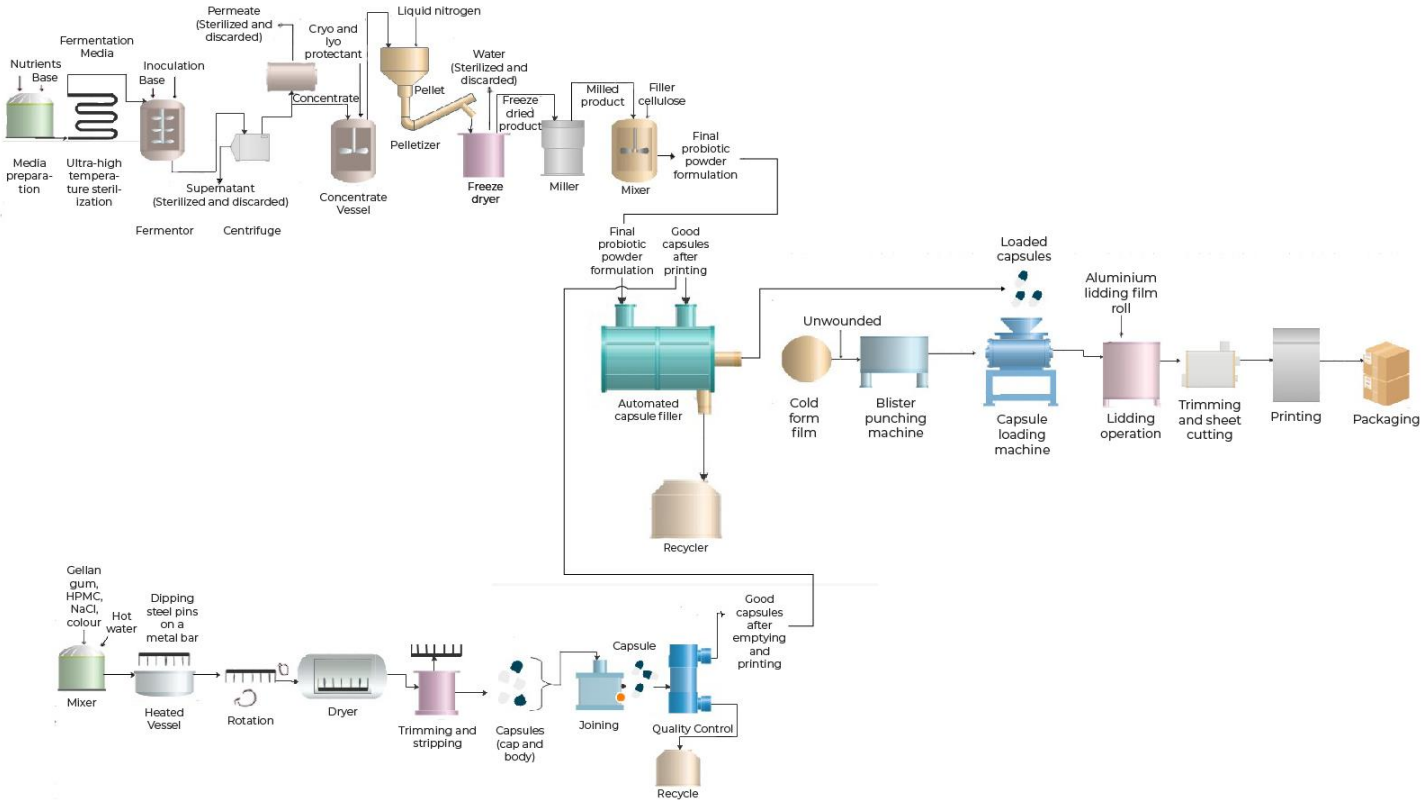
Packaging flowchart

6. Recycling and waste management

In few of the processes involved, there will be recycling of the defective and/or by products. Other by products and/or discards which cannot be recycled will be disposed in an effective and eco-friendly way.

The permeate, filtrate and the water as the by product from the freeze drying process in the powder manufacturing will be sterilized so that no recombinant bacteria is present, chemically treated to remove all possible metabolites and then will be discarded

The discarded capsules which do not cross the quality control process will be recycled.



Full flowchart

7. Glossary

Term	Definition	Ref
Allergen	An allergen is any substance (antigen), most often eaten or inhaled, that is recognized by the immune system and causes an allergic reaction.	Link
Antibiotic resistance	Antibiotic resistance happens when germs like bacteria and fungi develop the ability to defeat the drugs designed to kill them. That means the germs are not killed and continue to grow.	Link
Biomass	Biomass refers to the mass of living organisms, including plants, animals, and microorganisms or, from a biochemical perspective, cellulose, lignin, sugars, fats, and proteins.	Link
Bioreactor	Bioreactor is defined as a vessel that carries out a biological reaction and is used to culture aerobic cells for conducting cellular or enzymatic immobilization.	Link
Capsule	Capsules include medication that's enclosed in an outer shell. This outer shell is broken down in the digestive tract and the medication is absorbed into the bloodstream and then distributed and metabolized in much the same way as medication from a tablet.	Link
Clinical Trial	A clinical trial is a type of research that studies a test or treatment given to people. Clinical trials study how safe and helpful tests and treatments are. When found to be safe and helpful, they may become tomorrow's standard of care.	Link
Cryopreservation	It is the process of cooling and storing cells, tissues, or organs at very low temperatures to maintain their viability.	Link
Endospore	An endospore is a dormant, tough, non-reproductive structure produced by a small number of bacteria from the Firmicute family. The primary function of most endospores is to ensure the survival of a bacterium through periods of environmental stress.	Link

Escherichia coli Nissle 1917	*Escherichia coli* strain Nissle 1917 is a Gram-negative microorganism with probiotic properties that has been successfully used for the treatment of intestinal inflammation, especially in patients suffering from ulcerative colitis.	Link
Excipient	Excipients are inert pharmaceutical ingredients that are used in product formulations.	Link
Genetic drift	Genetic drift describes random fluctuations in the numbers of gene variants in a population. Genetic drift takes place when the occurrence of variant forms of a gene, called alleles, increases and decreases by chance over time.	Link
Inoculum	A small amount of material containing bacteria, viruses, or other microorganisms that is used to start a culture.	Link
Lidding material	Lidding materials and products have removable or hinged covers and are placed on the top of a container to secure its contents.	Link
Lipids	Lipids are molecules that contain hydrocarbons and make up the building blocks of the structure and function of living cells. Examples of lipids include fats, oils, waxes, certain vitamins (such as A, D, E and K), hormones and most of the cell membrane that is not made up of protein.	Link
Metabolite	A metabolite refers to any substance involved in metabolism. It is often regarded as the immediate by-product of a metabolic process. However, some references consider those involved in a metabolic reaction (not necessarily a by-product) as a metabolite.	Link
Pathogen	A pathogen is defined as an organism causing disease to its host, with the severity of the disease symptoms referred to as virulence.	Link
Probiotic	Probiotics are live microorganisms that are intended to have health benefits when consumed or applied to the body. They can be found in yogurt and other fermented foods, dietary supplements, and beauty products.	Link

Recombinant	Recombinant DNA (rDNA) molecules are DNA molecules formed by laboratory methods of genetic recombination to bring together genetic material from multiple sources, creating sequences that would not otherwise be found in the genome.	Link
Selectable marker	Selectable marker genes are conditionally dominant genes that confer an ability to grow in the presence of applied selective agents that are normally toxic to plant cells or inhibitory to plant growth, such as antibiotics and herbicides.	Link
Strain	A strain is a genetic variant, a subtype or a culture within a biological species.	Link
Viscosity	Viscosity is a measure of a fluid's resistance to flow. It describes the internal friction of a moving fluid. For liquids, it corresponds to the informal concept of "thickness": for example, syrup has a higher viscosity than water.	Link

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