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In-Situ Product Recovery Methodologies Practiced in Pharmaceutical Industries: A Review

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Abstract: *The demand of medicinesand pharmaceutical products is increasing more than ever and hence one needs to produce better products at lower cost. This can be achieved by using In-Situ Product Recovery (ISPR). This review paper attempts to provide a comprehensive understanding of ISPR and categorize and develop a generalized route to follow while prioritizing ISPR unit process for a particular type of product based on the properties of the product, type of system, economics constraints, scale of the plant etc. The unit operations under study with reference to ABE (Acetone- Butanol – Ethanol) and Lactic acid production via biocatalytic route are Gas stripping, Solvent extraction, In-Situ Product Crystallization, Adsorption, Perstraction, Pervaporation, Extractive fermentation, Vacuum fermentation. The ISPR method address these limitations by removing the product from the vicinity of the biocatalyst as soon as the product is formed thereby increasing the reaction rate and avoiding catalytic poisoning due to product itself. It can also provide further benefits for the subsequent downstream processing. This method operates as an integrated system that carries out multiple unit operations at a time.*

Keywords: *Downstream Processing, Catalytic poisoning, ABE Fermentations, Lactic acid, Biocatalyst.*

1. INTRODUCTION

Medications be it allopathic or herbal or any other form are essentially chemicals and the processing is somewhat similar with the exception of the starting material. Drugs are classified as fine chemicals as they as their effectivity is a parameter of merit of the drug rather than their chemical composition as in is in case of bulk chemicals (like hydrochloric acid, sulphuric acid, EDTA etc).

Manufacturing of pharmaceutical products at low cost is in demand today. This cost reduction can be achieved either by using cheap raw materials or by optimizing the production process. Now this optimization can be of many types like supply chain optimization or proper energy audits or increasing the product separation efficiency.

The supply chain of pharmaceutical industries has the following stages:

1) Primary Manufacturing
2) Secondary Manufacturing
3) Market Warehouses
4) Distribution Centre
5) Wholesalers
6) Retailers /Hospitals.

Now in case of primary manufacturing we deal with the processing of the active pharmaceutical ingredients (API). Cost can be reduced if API processing efficiency can be increased. Compared to chemical processes, a biotechnological process using whole cell biocatalysts is characterized by low productivity because of inhibition at product concentrations. Furthermore, the product stream is dilute, which leads to high costs in the subsequent isolation and purification of the product. The performance

of biotechnological processes can be enhanced by either strain improvements (screening of mutants, recombinant DNA technology) or by process engineering solutions. [1]

The most common approach to raise the productivity of a biochemical process is to increase the cell concentration in the fermenter or to remove the toxic product from the broth [2].

In any product manufacturing there are two types of processing.

1. Upstream processing
2. Downstream processing

Upstream processing: It refers to the first step in which biomolecules are grown, usually by bacterial or mammalian cell lines, in bioreactors. When they reach the desired density (for batch and fed batch cultures) they are harvested and moved to the downstream section of the bioprocess. The main objective of upstream processing is to create the environment necessary for cells to make the target protein. The protein serves various medicinal purposes [3]. It can also be referred to as the type of processing in which the production formation takes place. For example, if we consider the case of fermentation of barn to make beer then the drying of grain, crushing, forming malt and addition of the contents to the fermentation tank are all a part of upstream processing while as extraction of beer from the broth and further distillation to produce the desired concentrated beer are all a part of downstream processing.

Downstream Processing: It refers to the recovery and the purification of biosynthetic products, particularly pharmaceuticals, from natural sources such as animal or plant tissue or fermentation broth, including the recycling of salvageable components and the proper treatment and disposal of waste [4].These products from biocatalytic reactions are recovered via downstream processing which has various units operations aligned, such a set up might be more energy intensive as compared to a combined system inclusive of two or more-unit operation. This integrated system is considered In-situ product removal (ISPR) [5].

The ISPR can increase the productivity or yield of a biochemical process by any of the following ways:

a) Overcoming inhibitory or toxic effects of product to allow continuous formation at maximal production level,

b) Minimizing product losses owing to degradation or uncontrolled release (e.g., by evaporation)

c) Reducing the total number of downstream processing steps. [6]

As stated by freeman and co-workers a product may be removed from its producing cell by five main possible techniques. [6,7]

1. Evaporation by gas stripping.
2. Distillation by vacuum or membrane techniques like pervaporation.
3. Extraction by using water immiscible organic solvents, supercritical fluids or membrane supported extractive method like perstraction or preferential solubility like counter-current chromatography.
4. Size selective permeation methods like dialysis, electrodialysis, reverse osmosis or nanofiltration.
5. Immobilization techniques like adsorption.

The techniques/ unit operation would be studied in detail with the help of two case studies:

1. ABE Fermentation
2. Lactic acid productions using extractive fermentation

2. HISTORY

Preliminary research on the application of ISPR techniques in biotechnology was done in the 1960s and 1970s for the on-line removal of a toxin by an aqueous two-phase system. Salicylic acid by ion-exchange resins, 7,8-epoxy-1- octene by extraction into a water immiscible solvent and ethanol by

vacuum fermentation. Dialysis was applied to remove lactic acid and cycloheximide. Large-scale fermentative production of lactic and citric acid was done by the addition of lime to precipitate the calcium carboxylates. Extensive study on the various ISPR techniques began in the early 1980s to increase the productivity of ethanol fermentation with respect to ethanol production in the petrochemical industry [8]. There has been a lot of reviews on ISPR techniques ever since in whole cell biochemical processing that had been published. There are also available papers on common compounds like ethanol or butanol.

3. CASE STUDIES

3.1. ABE Fermentation

The main component to be extracted is butanol which is used in drug manufacturing for antibiotics, hormones, and vitamins. Traditionally, biobutanol is prepared when C. acetobutylicum decomposes carbohydrates like starch and glucose to acetone, butanol and ethanol. The ABE fermentation produces solvents in a ratio of 3 parts acetone, 6 parts butanol to 1 part ethanol. The reaction is biphasic i.e., during the first stage there is acid formation (acidolysis) followed by solvent formation (solvolysis). It can be inferred that the process is greatly affected by the pH of the system, media composition, redox state, initial substrate concentration. During this process butanol hinders the reaction by acting as a toxic as the structure of the substrate and product are similar and it occupies the biocatalytic sites thereby reducing the overall efficiency. The main reason to opt ISPR is high substrate cost and high energy demand for separation of the product. A number of ISPR techniques have been studied that were applied for the product recovery.

3.1.1. Gas Stripping

Gas stripping is a separation technique that involves the removal of solvents via dissolution into a gas passing through the fermentation broth. This technique was studied by a range of authors from the mid-1980s (e.g., Ennis et al) [6].

In this case a recycled mixture of carbon dioxide and hydrogen was sparged through the fermenter from the bottom, the solute was stripped off with the incoming gas and the later the vapour phase was condensed to recover solute (in this case butanol) while as the gas mix was recycled. This particular method can be performed in situ without the need for expensive equipment and plant modifications, gas stripping is considered a simple technique but there are certain adverse effects like Based on data from Ezeji et al, the concentration in the gas stream is very dilute, which implies that large condensing duties will be required, which will significantly increase operating costs. Additionally, the compressor duty to supply gas at high flow rates for a plant-scale reactor is energy intensive [9]. Other factors to consider were prevention of excessive foaming of the broth that might have a negative impact on the microbes, need for large volume of reactors to carry out gas stripping. It was also observed by Ezeji [10] that the process in continuous mode had less productivity as a result of reduction in the nutrients (substrate itself) if it is carried along with the gas. Heavy gas stripping may also result in pH fluctuation due to nutrient imbalance. A general conclusion to be drawn from the data generated by Ezeji and analysed by Stark is that the productivity of the fermentation is improved through the application of gas stripping.

3.1.2. Vacuum Fermentation

In this technique the pressure is reduced across the fermenter so that the volatile material is stripped off the top. The viability of vacuum fermentations was experimentally tested by Mariano *et al*. Mariano *et al* demonstrated that it is possible to recover ABE from fermentation broths under vacuum on a laboratory scale with no adverse effects on the bacteria [11]. It was reported that under constant vacuum conditions, the rate of removal of butanol was approximately 10 times higher than that found by Ezeji *et al*. Two vacuum modes have been investigated: constant and cyclic. Cyclic vacuum fermentations were found to be considerably more competitive in terms of energy demand than traditional distillation. The cyclic vacuum process allows the concentration of butanol to build up, then reduces the concentration rapidly by applying a vacuum for 2 h, repeating this process throughout the fermentation [12]. And the selection depends on the type of operation. One disadvantage of this method is it alters the pH balance of the overall process by reducing the overall content of butanoic acid in the broth.

3.1.3. Perstraction

It is a derived from liquid extraction methodology. It works on the same principles of mass transfer of ABE from the aqueous phase to an organic solvent, only difference being that the organic solvent and fermentation broth are separated by a membrane. The ABE transfers across the membrane into the organic phase. It is very similar to pervaporation, but has a liquid on the permeate side to provide the "driving force" rather than a gas or vacuum.The majority of research has used silicone tubing as the membrane. IT is a membrane driven process and the selection depends on the properties of the products to be extracted. For instance, if the product is corrosive of scaling, one would not opt for this method despite its high effectivity. [13]

Pervaporation: It also uses membrane across the two phases and the driving force here is generated by applying vacuum across the membrane unlike perstraction where the driving force is solvent affinity. The choice of membrane is of crucial importance here. This method is utilized for batch as well as continuous process. The product isolation is effectively carried out by this technique [14].

3.1.4. Adsorption

It is the oldest technique investigated for the use of ISPR from ABE fermentations. A wide range of adsorbents have been used in conjunction with the butanol and the ABE fermentation, and this list is continually evolving as new, more complex adsorbents become available. Some of the adsorbents used are activated carbon, silicate or silicate based zeolites, and polymeric resins [15]. A downside of adsorption is that it is inherently a batch process, as the ABE has to bind to the adsorbent and then, once it has reached capacity, desorption needs to take place to regenerate the bed [16]. In many experiments, batch adsorption was performed, meaning that once the adsorbent has reached capacity it can no longer relieve product inhibition. This indicates that the ratio of adsorbent to broth needs to be optimized, as the productivity varies with the quantity of adsorbent [15].

The alternative is operating a minimum of two external packed bed columns in a cyclic manner, allowing for one column to be adsorbing, while the other is desorbing. Operation in this cyclic manner with a fed-batch fermentation yielded a favorable fermentation productivity. This operating mode would reduce the adsorbent inventory required per fermentation, although the product removal would have to occur externally from the bioreactor [17]. Ideally, the development of a continuous adsorption process (e.g., simulated moving bed adsorption) would be best suited, to allow the simplest removal of ABE and regeneration of adsorbent. Once the adsorbent has reached its capacity the ABE needs to be removed and the adsorbent regenerated

3.2. Lactic Acid

Itis used in parenteral/I.V. solution,dialysissolution,mineral preparations, tabletcoatings, prostheses, surgical, sutures, controlled drug delivery system. Lactic acid or 2-hydroxypropanoic acid is an important commodity used in the food, chemical and pharmaceutical industries. Interest has grown in using lactic acid as a monomer in the production of biodegradable and biocompatible polylactic acid (PLA) polymer [18]. The process of lactic acid formation involves two specific pathways hydrolysis of lignocellulose biomass to form pentose which is further fermented to for lactic acid or Enzymatic hydrolysis of cellulose to form glucose which is fermented to lactic acid. In either of these pathways the Conventional lactic acid fermentation usually has an end product inhibition effect, which causes the decrease in productivity when using high substrate concentrations [19]. Various techniques have been investigated to overcome such limitations, and extractive fermentation(ISPR) provided a promising result.[20-21].

3.2.1. Extractive Fermentation

The process begins with preparation of anion exchange resin which in placed in the fermentation broth to adsorb lactate. This resin now is separated by sieving and then put through three steps—washing, eluting and regenerating— before being reused in other batches of fermentation. After eluting with HCl the lactic acid in water solution undergoes ISPR / separation. the eluted solution from the resin—were extracted by organic extractants. Extractive fermentation employing an anion exchange resin for in situ removal of lactic acid during the fermentation of L. lactis ATCC 11454 provided high productivity for lactic acid compared with conventional batch culture. Using this technique, high substrate (glucose) can be used with a much lower inhibitory effect from the lactate

produced [22]. The experiments analysed by Malliak showed that the extracted lactate by conventional method was about 23g for 77g of biomass in broth while as the lactate extracted by the resin bound method was about 29g [15]. This indicates the product recovery of about 6g per 77g of biomass.

4. OTHER METHODS

4.1. Counter Current Chromatography

Is a form of chromatography using two immiscible liquid phases (rather than a conventional solid phase ligand support) to separate solutes on the basis of their relative solubility in the two solvents [24]. The pairs can be aqueous–aqueous, aqueous–organic or organic–organic. The technique, which is essentially an intensive liquid–liquid extraction process, allows chromatographic-quality separations but with a much greater capacity than conventional solid adsorbents [25]. It can be applied to a wide range of purification applications, including natural-product isolations (e.g., antibiotics and metabolic intermediates) and fine-chemical separations.

The principle of operation of devices used for CCC is as follow. An inert plastic or stainless-steel tube (internal diameter typically 1–3 mm) is first wound around a drum. The drum is then rotated in a planetary, epicyclic motion around a stationary (or sun) gear. During the period of rotation, each point within the coil will experience an alternating acceleration field with the greatest force occurring at the furthest point from the centre of rotation. The fluctuating fields will establish alternating zones of phase mixing (low acceleration force) and phase separation (high acceleration force) of the two immiscible liquids in the coil. This repeated action of phase mixing and separation is typical of a conventional equilibrium-stage separation process except that, in this case, owing to the motion and speed of rotation of the coil, thousands of stages can occur in a single machine [26].

Fractionation of a sample is usually achieved by pumping the less-dense mobile phase through the coil, where it is repeatedly contacted with the denser stationary phase (which is retained within the coil owing to its rotation). The mixture to be separated is introduced into the mobile phase and the fractionated components emerge from the end of the tube in the order of their partition coefficients [27].

The quality of fractionation that can be achieved depends upon several factors including the physical and chemical properties of the two phases (e.g., density, viscosity, polarity), the properties of the coil (e.g., construction material, internal diameter, length), the mobile-phase's flow rate and the mechanical design of the instrument (e.g., drum radius, sun-gear radius, rotational speed). The machine is also versatile in its mode of operation. Either of the two solvent phases can be used as the stationary phase, and the mobile phase can also be switched during operation so that highly retained solutes can be eluted from the coil [28].

A key design requirement for ISPR applications of this technology will be to match the solvent characteristics for selective elution with those already established for extractive biocatalysis. Unlike conventional HPLC, CCC machines can handle crude feed materials containing particulates, although phases with a tendency to emulsify can cause operational problems resulting in the stationary phase overflowing as emulsified droplets [29].

4.2. Molecular Imprinting

It is an emerging technique in which polymeric adsorbents are synthesized that exhibit highly selective binding for a particular target molecule. Current imprinting strategies rely on either covalent or noncovalent interactions between the target molecule and the polymer during the imprinting and adsorption stages, or on a combination of both [30]. The noncovalent approach is the easiest and most widely used methodology to date. MIPs have been shown to discriminate between stereoisomers and between different molecules on the basis of a single hydroxyl group [31], and are therefore of interest as a highly selective ISPR tool. An added benefit is that molecular imprinting is a potentially generic separation technique, as shown by the wide range of product categories (e.g., pharmaceuticals, pesticides, amino acids, carbohydrates, dyes and metal ions) to which it has been successfully applied [32]. The known characteristics of MIPs can be compared with the desired criteria of solid adsorbents for use in ISPR applications. Although the selectivity and mechanical and chemical stability of the polymers are excellent, their major drawback is currently their low capacity for the target molecule.

Ongoing research on the rational design of functional monomers should improve this in the near future. A major increase in capacity, together with the intensive application and reuse of the adsorbents, might lead to their eventual introduction in industrial processes. MIPs have recently been used as an ISPR tool to overcome an unfavourable reaction equilibrium in the synthesis of the artificial sweetener aspartame [33]. Enzymatic condensations of benzyloxy carbonyl-L-aspartic acid (Z-L-Asp) with L-phenylalanine methyl ester (L-Phe-O-Me) carried out in the presence of aspartame imprinted polymers increased the yield of aspartame by a factor of more than four. To synthesize molecularly imprinted polymers (MIPs) using the noncovalent approach, polymerizable monomers containing functional groups complementary to the target (template) molecule must first be selected [34]. This selection depends upon the chemical functionality of the target molecule and whether hydrogen bonding or electrostatic or hydrophobic interactions are to be exploited in the solvent used for rebinding of the target molecule.

4.3. In-Situ Product Crystallization

The use of in situ crystallization as a form of bioprocess intensification targets the removal of a synthesized product directly from its reaction solution in some form of a solid state. This is typically referred to as RC, reactive precipitation, or ISPC in biocatalytic reactions.

In situ crystallization effectively leads to an apparent shift of the chemical equilibrium towards the product side, which directly results in higher yields and productivities of a given (bio-)process. Notably, the crystallization of such a solid product form is fortunately a simultaneous purification of the product, since it usually crystallizes in a relatively pure form and can be easily filtered from the reaction mixture [35].

There are two types of IPSC which are:

1. Induced crystallization.
2. Spontaneous crystallization.

Process options featuring an ISPC are divided into internal and external crystallization. [36] Internal crystallization includes product crystallization directly within the reactor, whereas external crystallization basically requires an external unit that facilitates crystallization outside the original reactor.[37].

5. SELECTION OF ISPR

It has to be based on two main criteria:

1. According to product properties
2. According to process constrains

Freeman and Co-workers proposed five principal product properties to help choose the most suitable ISPR techniques.

1. Volatility
2. Hydrophobicity
3. Size
4. Charge (positive, negative, neutral)
5. Specific binding properties of a compound

can be used to group and assign the products to their appropriate ISPR methods.

The chart developed by Stark in his review of ISPR in whole cell biotechnology during last twenty years summarizes the selection based on product properties.

The selection of the process is also based on the physical and chemical properties of the product. To extract butanol from fermentation broth one needs to check for the solubility of butanol in the mix and other properties like hydrophobicity, charge, size. If butanol is soluble extractive fermentation can be used or else one can prepare a supersaturated solution and use reactive crystallization (In-Situ product crystallization). If butanol it is sparingly soluble or the distribution coefficient is approaching towards unity one could opt other methods like gas stripping, Hybrid gas stripping, Perstraction etc.

This chart though provides plausible selection criteria but however fails to analyse the system and thus has a wide pool of methods which can be obtained for similar product isolation [38].

This help to further narrow the scope which in turn guides to choose proper ISPR as per the product and process parameters. This can be understood by considering the case of ABE fermentation. In this the gas stripping, vacuum fermentation and adsorption are all suitable and gives improved results but for instance we take into mode of operation which here is continuous then method like adsorption is not really good option as one will have to change the adsorption beds often and that might consume more time thereby resulting in reduced productivity.

Hence, we need Process constraints like:

1. Mode of operation
2. Contact type (Direct/ Indirect)
3. Removal type (Internal / External)
4. Market demand of the product v/s associated revamping changes
5. Scalability

ISPR Technique	Product categories: Low molecular wt					High molecular wt			Important examples
	Hydrophobic – volatile	Hydrophobic – nonvolatile	Hydrophobic – neutral – volatile	Hydrophobic – neutral – nonvolatile	Hydrophobic – charged	Hydrophobic	Neutral	Charged	
Evaporation									
stripping, (vacuum-) distillation, per vaporation, transmembrane distillation	++		++						ethanol
Extraction									
organic solvent (inc. perstraction)	++	++	+	+		+			ethanol, flavors
supercritical fluid	+	++	+	+	+				flavors
reactive (inc. perstraction)	+	+	+	+	++	+	+	+	organic acids, ethanol
aqueous two-phase			+	+	+	+	++	++	enzymes
Permeation									
dialysis			+	+	+				lactic acid,ethanol, ammonium
electrodialysis					++				lactic acid
reverse osmosis, nanofiltration			+	+	+				ethanol
Immobilization									
hydrophobic adsorption	++	++	+	+		+	+	+	butanol, flavors
ion-exchange					++			+	organic acids
affinity adsorption	+	+	+	+	+	++	++	++	proteins
Precipitation					+			+	lactic acid, citric

Fig5.1. *Selection of ISPR.[1]*

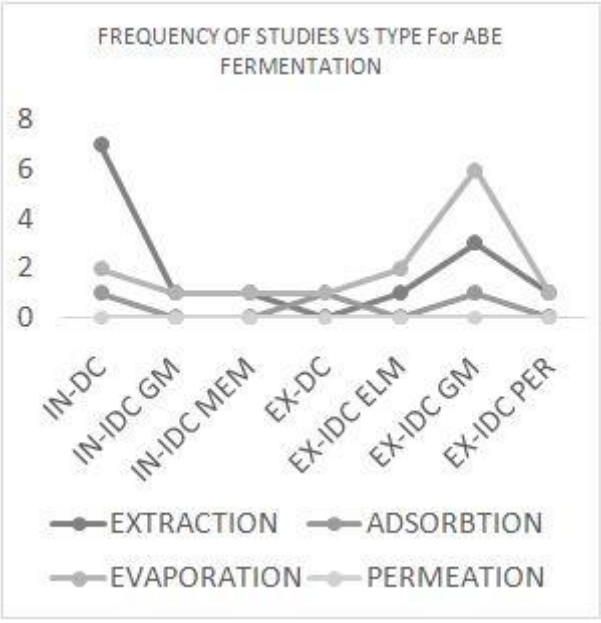

Fig5.2. *Cases for ABE Fermentation*

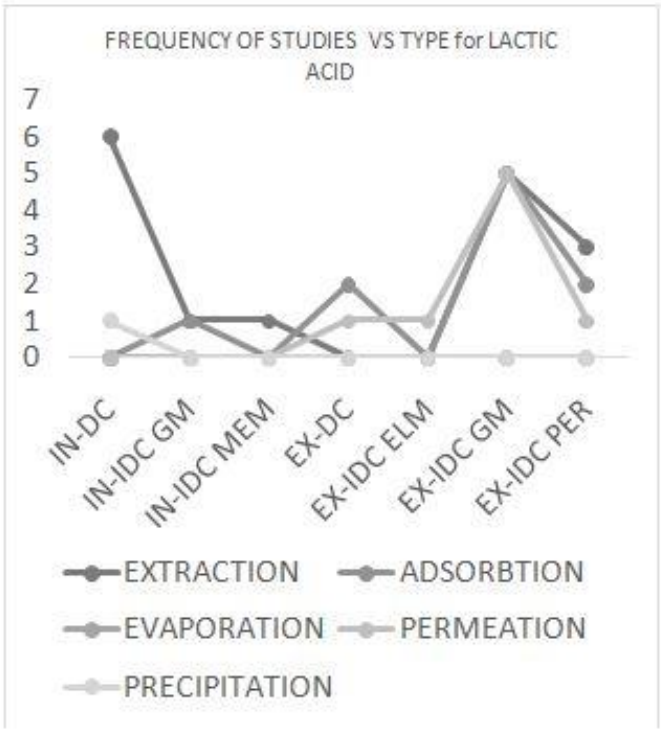

Fig5.3. *Cases for Lactic Acid*

Notations:

IN-DC – In – situ Indirect contact

IN-IDC-GM – In-situ Indirect contact gel matrix

IN-IDC MEM – In-situ Indirect contact membrane

EX-DC – Ex -situ direct contact

EX-IDC-GM – Ex- situ indirect contact gel matrix

EX-IDC-PER- Ex- situ indirect contact pervaporation.

The charts represent the number of studies conducted on the ISPR method to improve the recovery ABE (in chart 1) and lactic acid (in chart 2) from past 20 years. The has been total 25 studies that had been conducted for increase in productivity using ISPR for ABE Fermentation using the bacteria *clostridium acetobutylicum*and a total of 30 studies on lactic acid by using *lactobacillus* since past 20 years. The graph clearly indicates that maximum studies has been carried out for in-situ direct contact indicating the prime suitability of operation of this method. The charts further narrow down the most common method chosen as ISPR technique to increase the overall yield of the process and avoid product poisoning [39].

6. Order of Selection

The order in which we prioritize the criteria can be summarized in a process map as follows:

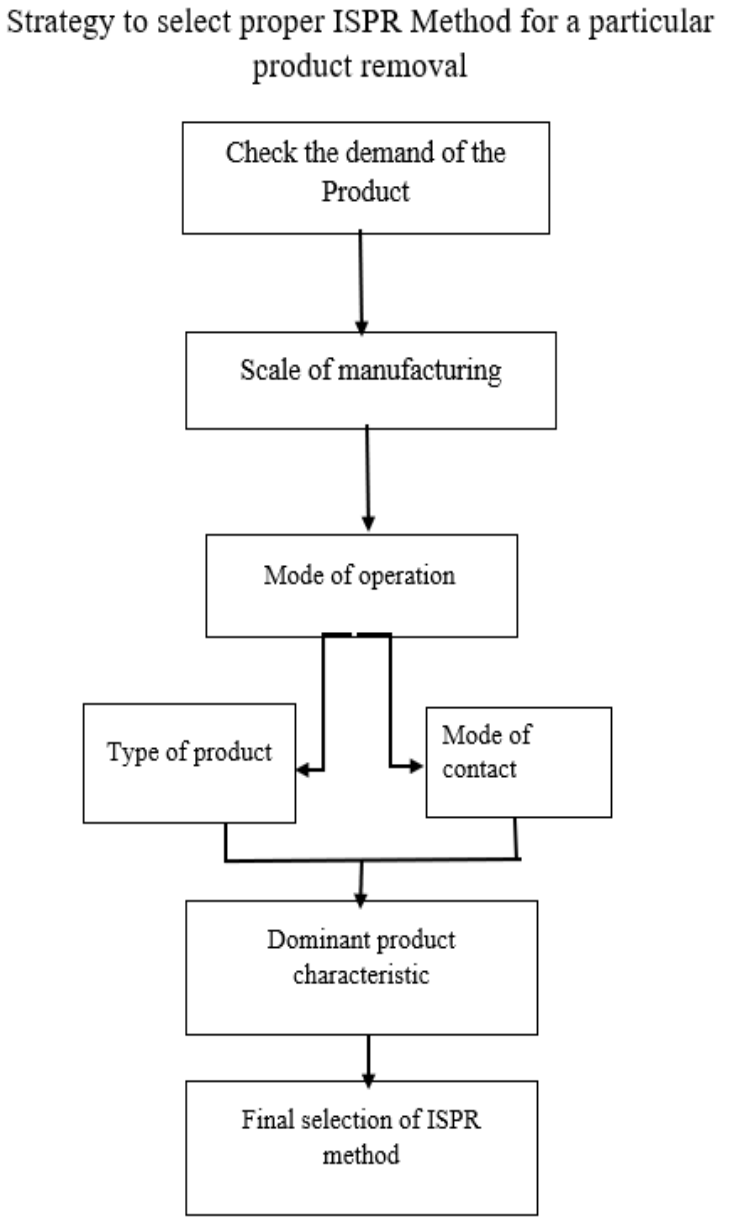

Fig6. *Order of Selection*

7. CONCLUSION

The need for steady supply of pharmaceuticals at lower rates generates the need for process optimization. This is where ISPR plays a vital role though the product recovery methods as discussed. Such techniques have their pros and cons and a general idea/methodology have been discussed on how to proceed. The techniques have been illustrated by taking the case studies of ABE fermentation and lactic acid production processes. A lot of new unit operations have been studied and their use in downstream processing has been emphasized. In situ recovery has an edge over conventional separation as this is done in the reactor media itself and thus is more effective and is also cost friendly as it negates the need of a new separation setup. The generalization on the techniques to be followed is though not accurate always and it needs to be changed according to the reaction conditions and should favour the equilibrium conditions. The general flow pattern to select proper ISPR will benefit to in case of deciding the priority of the process constraint.

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