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Chapter 1

Advances in Cancer Immunity, A Formidable Army

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1. Introduction

Cancer is a group of diseases involving abnormal cell division in an uncontrolled manner. Cancer affects almost all tissues; probably there are more than 200-250 types of cancer including breast cancer, ovarian cancer, skin cancer, leukemia etc. Different cell types in our body that can undergo the changes associated with cancer result in one or the other type of cancer [20].

Cancer cells acquire alteration in their genes which allows these cells to proliferate abnormally and make more copies of itself than a normal cell can and forms a compact mass of cells. These cells might look identical to its neighbors, but there are too many changes like over growth of tissue, a process called hyperplasia. Within this collection of cells, the cells divides more rapidly and abnormally, pile up on one another and there is a loss of contact inhibition. This is the development of the early stage of the tumor, a benign tumor or Adenoma. Within this mass of cells, further alterations may take place. Tumor cells accruing blood vessels into the tumor to nourish it and bring the growth factors to tumor that are required for their survival. In addition, these cells degrade the extracellular fluid also acquire the ability to move away from the initiation site and leave the primary site to disseminate throughout the body, creating a secondary tumor. This happens when cells access the blood vessels and take up residence in the secondary site, this is called metastasis.

2. Genetics of Cancer

In normal cell cycle, the cell goes from mitosis to G1 [gap1] phase, here cell increases in size and prepares itself to copy its DNA. The replication of DNA in the next phase is termed 'S' phase. Once the chromosomes replicate, the cell enters second gap phase G2, it is a period of protein synthesis and rapid cell growth where cell prepares itself for mitosis or M phase. In M phase cell growth stops and cell is ready to complete cell division and produce its two daughters. The new daughters immediately ensue into G1 phase and the cycle continues. It is a well regulated process.

There are basically two types of mutations that occur in the genes of tumor transformed cells.

1. Acquired mutation: Acquired mutation is not hereditary. It cannot pass from generation to generation. In this type of mutation, genes are damaged by ultra-violet rays, tobacco smoke and other factors like age, virus, dietary carcinogens and environmental carcinogens.

2. Germ line mutation: In Germ line mutation, mutation occurs in the reproductive cell. So it transmits from parents to a child.

There are different types of genes linked to cancer. E.g.: protooncogenes, oncogenes, tumor suppressor genes etc. protooncogenes are those which helps in normal cell proliferation and growth. The protooncogenes code for the protein which stimulates the cell division. Normally these genes encode proteins when growth factor is available. Mutation in these protooncogenes results in conversion into oncogenes and cell grows uncontrollably. For instance, a mutation in Ras protein makes it oncogenic, resulting in stimulation of cell division even when no growth factor around.

Tumor suppressor genes regulate the cell growth or slow down the cell division, repair DNA mismatch and apoptosis. Mutation in tumor suppressor genes results in loss of trait that is their ability to limit cell growth. Eg: p53 genes, pRB genes (protein retinoblastoma genes), p21 genes. pRB gene and p53 genes act as checkpoints between G1 and S phase. pRB genes block the transition from G1 phase to S phase in its active form. It can be inactivated by phosphorylation through kinases which is stimulated by growth promoting signals. p53 plays a crucial role in cellular response to DNA damage. It binds to damaged DNA and trigger cell growth arrest or apoptosis. However, in cancer cell defective in p53, it is unable to binds the damaged DNA and results in damaged DNA passes on [5,8].

Normal cell division is regulated by stop signals. Cancer cells have defects in these classes of stop signals. Because of alteration in Ras (proto-oncogenes), cancer cells are more capable of dividing because Ras protein plays an important role in controlled cell growth,

proliferation and migratio. Moreover, Mutation in tumor suppressor genes like pRB genes and p53 genes the stop signal is misprocesses or completely lost and cell continue to divide.

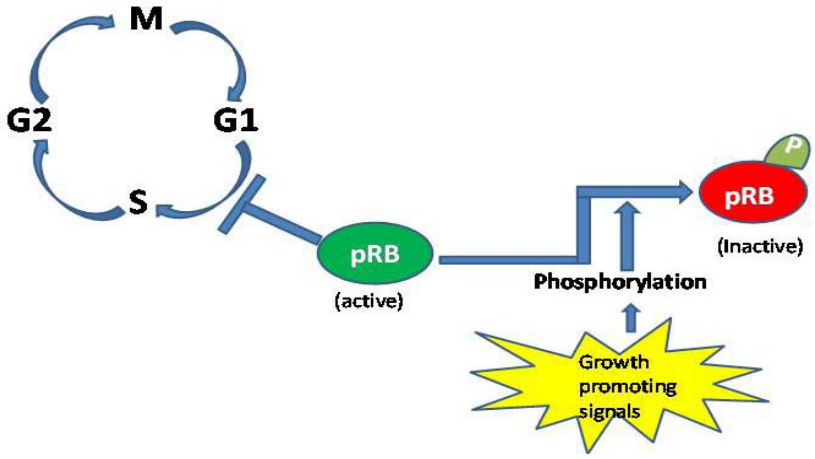

Figure1: Growth promoting signals phosphorylate pRB and inactivate it.

3. Immune Cells in Tumor Microenvironment

Our immune system is the body's defense against the infected and tumor transformed cells. Thus it is imperative to understand the role of our immune system and its responses in tumor formation and development and also the suppression of tumors.

Traditional immune responses like immune surveillance and immunoediting are crucial for preventing and inhibiting tumor development, for example, CD8+ and CD4+ Th1 cells along with cytokine IFN-γ are major anti-tumor immune effector cells. On the other hand, more current research show that incomplete responses can, in fact, promote growth and progression of cancer, particularly responses like inflammation. The convoluted nature of the tumor microenvironment is delineated by the elucidation of different subsets of immune effectors and regulatory cells. Tumor-induced effects on the differentiation and functioning of cells make this unique environment even more variable [2].

3.1. Dendritic cells

Dendritic cells are the most potent and well-known antigen presenting cells (APCs). Dendritic cells identify, process and present different tumor antigens to specific T-cells. They also maintain both, innate and adaptive immune responses by networking with myeloid and lymphoid cells. Immunohistochemistry shows that infiltration of DCs into primary tumor lesions has been associated with reduced incidents of metastasis, delayed tumor progression and lengthened patent survival.

Additional data support that the maturation state of DCs has diagnostic relevance. For instance, IHC analysis of the density of CD1a expressing DCs and the maturation marker DC-LAMP in cutaneous malignant melanoma shows that CD1a+ DCs were detected both in infiltrating melanoma cell nests and in the surrounding stroma, while DC-LAMP+ mature

DCs were mostly confined to the peritumoral areas. The degree of infiltration by CD1a+ and DC-LAMP+ DCs was inversely proportional to the thickness of melanomas and the high peritumoral density of mature DCs was associated with prolonged survival, simultaneously, the density of CD1a+ cells had a prognostic impact [11].

Programmed cell death in DCs plays an essential role in the regulation of immune responses and elimination of DCs from the tumor microenvironment significantly impacts the efficacy of anti-tumor immunity and facilitates tumor escape from immune recognition. Research has shown that a multitude of cancers, for instance, the apoptotic rate of TIDCs in endometrioid adenocarcinoma has been reported to be particularly higher than in normal endometrium. Many tumor-derived factors, including gangliosides (GM3 and GD3), neuropeptides, and other molecules are prominent inhibitors of DC function and known to induce apoptosis of DCs. DCs in tumors lose (or have limited) their ability to present tumor-inducing cells and induce the proliferation of tumor-specific CD4+ and CD8+ T cells. Studies also reveal abnormalities in the form of reduced production of IL-12, suppressed endocytic activity, inhibited antigen-processing machinery, abnormal motility, etc [11].

On the other hand, several molecules and signaling pathways, including the production of IL-10 and TGF-β, expression of IDO, iNOS and arginase, or expression of inhibitory B7-related molecules, play a role in immunosuppression by regulatory DCs. For instance, the interaction of B7-H1 with PD-1 on tumor-infiltrating T cells is a widely cited theory of immune suppression involving B7-H1 in ovarian cancer, PD-1+B7-H1+ DCs have a classical DC phenotype, but are immature, suppressive and respond poorly to danger signals. T cell suppressor function of these DCs appeared to be mediated by T cell-associated PD-1

Thus, the tumor milieu, controls functional polarization of DC differentiation and activity, as well as their ability to interact with other immune cells. Simultaneously, the network of immunosuppressive DCs are a critical part of supporting tumor progression and restricting the success of different therapeutic modals in the cancer patient.

3.1.1. NK Cells

NK cells are lymphocytes found to origin in the bone marrow. They are a part of the innate immune system with the capability to kill tumor cells upon activation. Once activated, they can follow two effector paths, first, they exocytose various cytotoxic granules containing perforins and granzymes also. These granules permeate the target cell and induce apoptosis. Alongside this, NK cells initiate the death receptor cascade. The NK cells interact with the TNF receptor superfamily, (FAS, TRAIL-R1, TNFR1, etc) by secreting their ligands. The second effector mechanism involves their ability to release a myriad of cytokines and chemokines, including INF-g, TNF, GM-CSF, MIP-1α and RANTES.

Extensive studies done in mice models show that those lacking NK cells have more aggressive tumor growth and metastasis. In human, clinical studies (in leukemia patients) have produced evidence for the benefits of NKCs. Implementation of these cells for anti-tumor strategy is done by the proper activation or inhibition. In humans, inhibitory NK receptors are members of the KIR family and in mice, of the C-type lectin-like Ly49 receptors, both sensing the expression of various allelic variants of classical MHC class I molecules. Other inhibitory NK receptors that engage non-MHC-encoded self-surface molecules for example, NKC-encoded CTLR KLRG1 (human and mouse), NKR-P1A (human) and Nkrp1d (mouse). While NCR NKp30, NKp44 and NKp46, and the CTLR NKG2D are prominent activating receptors [18,19,20].

3.1.2. Tumor -associated Macrophages

Macrophages, phagocytosing immune cells which are distributed in all tissues, macrophages are well recognized for their roles in homeostasis, tissue repair and development. One area of research on macrophages is of particular interest, tumor associated macrophages (TAMs).TAMs are myeloid derived suppressor cells (MDSC), which play a critical role in tumor progression in the tumor environment [10]. They augment cell proliferation, invasion, and metastasis; promote angiogenesis and hamper anti-tumor immune response. At initial stages, M1 cells infiltrate, activate and release pro-inflammatory cytokines and chemokines (CXCL19 and CXCL10) which in turn attract Th1, Th17 and NK cells. However, in advance tumors TAMs are polarized to form M2 cells, which release CCL17, CCL22 and CCL24, they encourage Th2 and Treg cell recruitment and differentiation. Thus TAMs can serve as tumor inhibitory as well as promote tumor development and immunoregulation.

Other MDSCs produce high levels of IL-6. Whereas MDSC would normally differentiate after migration, the factors within the tumor microenvironment prevent differentiation and instead promote expansion and activation of the immature myeloid cell population that may result in the suppression of tumor immunity [20].

3.1.3. T cell response in tumor environment

- **CD4+ and CD8+:** T-cells are commonly known as tumor infiltrating Lymphocytes. Research elucidate that infiltration by these cells is considered a positive prognostic factor for initial stages of cancer. CD4+ and CD8+ cells release IFN-γ which has an important role in inhibiting tumor growth and killing tumor cells [4,20].

- **Treg Cells:** Regulatory T cells comprise a subset of immunosuppressive cells that aid in maintaining immune homeostasis and self-tolerance, thus promoting immune evasion and tumor progression.For example, in pancreatic and breast cancer patients, the prevalence of CD25+ regulatory T cells is visualized in the blood at much higher rates than that found

in normal donors. In cervical and cancer patients, functioning CD25+ regulatory T cells have been identified within the tumor draining lymph nodes.

Tregs infiltrate cancerous lesions or tumor-draining lymph nodes (TDLN) and get activated by TAAs and tumor-derived factors. These activated Tregs downregulate the anti-tumor activity of, NK cells and DCs, and secrete immunosuppressive molecules. Tregs are also known to promote and establish sites for metastasis. Thus Tregs are an intensively researched sites for immune targeting [1,20].

- **Th17 cells:** Th17 cells (T helper cells) and their effector cytokines (IL-17A, IL-17F, IL-21, and IL-22) maintain host defensive mechanisms against various infections and pathogens, especially extracellular bacterial infections, and are involved in the pathogenesis of many autoimmune diseases. TGF-β and IL-6, through activation of Stat3, signal the differentiation of CD4+ cells into Th17 cells and IL-23 (a pro-carcinogenic cytokine) maintains and propagates the inflammatory cell population. Thus Th17 cells act as an antagonist of IFN-γ, restricting their differentiation and tumor suppressing function [14,20].

3.1.4. Regulatory Cytokines

- **IL-6:** IL-6 is an integral cytokine which is known to encourage cancer cell proliferation and simultaneously inhibiting their apoptosis by activation of transcription 3 (Stat3). It also influences differentiation, in the presence of other cytokines as discussed above. It has shown to play an important role in carcinogen propagated liver cancer development. It has been implicated in many of the processes that involve TNF which itself plays an essential role in several cancers.

- **IL- 10:** IL-10 also plays a significant role in growth and maintenance of cancer cells, especially those derived from CD25+ Tregs. It downregulates inflammation and can also inhibit activation of NF-kB, however, it can activate Stat3 also, hence playing a key part in cell proliferation and survival.

- **TGF –β:** TGF-β is widely recognized as an immunosuppressive cytokine, usually inhibiting immune responses anti-cancer. Moreover, TGF-β plays an important role in initiating generation and functioning of CD4+ CD25+ Tregs under particular conditions. For instance, TGF-β suppresses IFN-γ production along with promoting the generation of Foxp3+ Tregs and the differentiation of Th17 cells, which together, favor the growth and proliferation of cancer cells. In conjunction, TGF-β is also a strong inhibitor of macrophage activation and reduces their signaling of inflammatory cytokines such as IL-6, TNF and IL-1b, which are aimed at impeding inflammation-associated cancer [20].

3.1.5. Inflammation

The inflammation acts to protect and isolate an infected or damaged area. However if the inflammation does not subside, evidence indicates that it can lead to tumor formation, growth and angiogenesis. Inflammatory cells produce Reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) cause mutations in neighboring epithelial cells. Furthermore, inflammatory cells release cytokines which elevate the intracellular ROS and RNI in pre malignant cells. This inflammation can lead to epigenetic changes that encourage tumor initiation. Cytokines synthesized activate NF-κB or STAT3 pathways in pre-malignant cells which promotes various pro-tumorigenic processes like survival, proliferation, growth, angiogenesis, and invasion. This forms a vicious circle as NF-κB and STAT3 further induce production of cytokines and chemokines that lure supplemental immune cells to support tumor-associated inflammation [3].

4. Therapeutics

The development and survival of cancer cell include several factors like a mutation in genes, many physiological alterations. The changes include loss of functions like alteration of tumor suppressor gene, mutation in oncogenes leading to rapid proliferation and immortality.

Mortality in cancer is fastidiously associated with metastasis of the tumor. Even though the primary tumor can be removed surgically there are always chances of remission due to metastatic tumor growth which might be lethal if unchecked or associated with vital organs such as brain or lungs. As conventional surgical treatment of tumor is not effective especially in the case of distant metastasis, there is a greater need for therapies involving the immune system of the body due to its specific nature and widespread reach.

The Anticancer drug can primarily be divided into four categories based on their mode of action. Chemotherapy is the first category as it involved a drug that induces cell lysis by either interfering with the synthesis of nucleic acid or negatively impacting the process of cell division. The second category is targeted molecule therapy which involves the use of molecules with high specificity for the target of choice. The third category consists of using a new kind of antibody with kinase inhibitors to block cellular signaling pathway important for cell proliferation by hormonal therapy. Lastly, immunotherapy involves the starting and aggravating anti-tumor immune response [16].

4.1. Immunotherapies

4.1.1. Vaccines

The concept of utilizing immune related cells was serendipitously discovered by William Coley in 1893, when he discovered that Streptococcus pyrogens infection led to spontaneous

remission in a patient suffering from sarcoma. This led to a series of experiments resulting in the discovery of Coley's toxins. The pair of toxins had high efficacy, even comparable to modern therapies.

Cancer vaccines work just like conventional vaccines, eliciting a lasting immune response. Just like early vaccines for infectious diseases, the old cancer vaccines made use of inactivated or killed cancer cells in the form of lysates and irradiated cells. These vaccines required no detailed knowledge about the intricate system of antigens. However, most resulted in quick relapse or low efficacy, therefore, this line of inquiry was not pursued further.

Most of the modern cancer vaccines utilize specific immunogen to elicit an immune response. Viral vectors are generally used for the vaccination due to their ease of disarmament and ability to induce strong cytotoxic T cell response. The second approach makes use of dendritic cells as they are prominent antigen presenting cells. The dendritic cells based vaccines skip the step from the transfer of antigenic peptide from the vector to the antigen presenting cell and directly present the antigen for detection by lymphocytes.

Both the vaccine types have been successfully tested for prostate cancer. However, the only FDA approved vaccine in the market is Sipuleucel-T manufactured by Dendreon for prostate cancer [17]. It contains monocytes collected ex vivo from patients and cytokines (GM-CSF) is used as an adjuvant.

4.1.2. Monoclonal Antibodies

Hybridoma technology enables the production high quantities of antibodies specific for a single antigen called a monoclonal antibody. In cancer therapy, monoclonal antibodies are used as a primary blocker of important antigens and ligands but can also be used as immune modulators.

Epidermal Growth Factor Receptor (EGFR) plays an important role in signal transduction for proliferation, migration and invasion utilizing the MAPK/KRAS signal transduction pathway. Several monoclonal antibodies such as cetuximab prevent the binding of the activator molecule (EGF) for EGFR, blocking the signal pathway. In a similar vein, human epidermal growth factor receptor 2 (HER2), is overexpressed in a quarter of cases of breast cancer and can be targeted by using Herceptin®. Both of the antibodies are FDA approved and in commercial use [17].

Despite the high efficacy of monoclonal antibody treatment, there have been cases of development of resistance against these antibodies as the cancer cells that do utilize the specific transduction pathway get aggressively selected.To circumvent the problem, a new strategy has emerged, wherein the inhibitors of CD-8 T cells, such as Cytotoxic T lymphocyte antigen 4

(CTLA-4) and Programmed Cell Death Protein 1 (PD-1), are being targeted for blocking by the monoclonal antibodies [12]. T cells are the major antitumor factor in immune response, thus increasing their population can increase the efficiency of the immune response. Other such drugs are also in development and seem promising

4.2. Chemokine Therapies

The introduction of immune stimulatory compounds in the vicinity of tumor cells leads to an increase in the potency of anti-tumor activity in the host. Due to such an immune stimulatory nature and their chemo attractant behavior of chemokines upon the white blood cell populations, a large number of studies are in effect to determine the extent of the impact of chemokine therapy in oncology. In monotherapy (using a single chemokine) studies it was determined that chemokines such as CCL -1, 2, 3, 5, 10, 16, 19 and 20, can mediate regression as well as increase immunity against future challenge.

Chemokines by themselves show little anti-tumor efficacy. However, when used in conjunction with other immune stimulatory chemo attractants IL-2 (T and NK cell activator) and XCL-1 (T and NK cell attractant), the efficacy is increased. The effects are primarily based upon the natural immune reactions involving $CD\text{-}4^{+}$ and $CD\text{-}8^{+}$ cells [7].

Another strategy involves fusing chemokine and tumor antigen using its immunooglobulin variable region for the fusion. It is predicted that the vaccine using the hybrid protein would generate enhanced protection against cancer [13].

Thus, chemokines alone and in conjunction with other molecules possess the capability of acting as immune cell attractant and decrease the tumor forming capability of malignant cancer cells. The combination is also a tumor suppressing agent. Lastly, chemokines might be used as adjuvants in cancer vaccines.

4.3. Adoptive Cell Therapy

Adoptive cell therapy is distinct from in vivo methods as a large number of T cells with desired epitopes can be generated in a short period of time in vitro that can later be selected for efficacy and specificity to effectively mediate cancer regression. In vivo growth allows for the production of T cells free from inhibitory factors that are a part of in vivo production.

Rather, it is a living therapy as the cells can proliferate after administration to continue on with their functions. However, one disadvantage is the identification of a cell that selectively targets the cancer cells while not affecting normal cells. ACT has mediated dramatic regressions in a variety of cancer histologies, like melanoma, cervical cancer, lymphoma, leukemia, bile duct cancer, and neuro- blastoma [15].

The current therapies make use of cells that are –

1. Native host cells with preexisting anti-tumor properties

2. Modified host cells made to express

i. Antitumor T cell receptors (TCR)

ii. Chimeric Antigen Receptors (CAR)

During the 60's little was known about T cells and their functions. A big leap in ACT development was the detailed description of IL-2 in 1976, which enabled the ex vivo culture of lymphocytes without a loss in function. Later in 1988, studies showed that adoptive transfer of tumor infiltrating lymphocytes (TILs) of autologous nature could help mediate regression in melanoma patients. This provided the first evidence that T Cells played a significant role in cancer immunotherapy for humans. However, the transferred cells had a short lifespan and disappeared within a few days of administration. The solution came in 2002, when it was demonstrated that non myeloablative chemotherapy, done before administration of autologous lymphocytes, not only increased the regression of cancer but also lead to a persistent proliferation of the cells within the host. However, melanoma appears to be the most effective TIL producer amongst cancer histologies, reliably giving rise to T Cells capable of expressing anti-tumor receptors. The continued interest in ACT leads to the development of genetically engineered lymphocytes modified to express anti-tumor receptors. In 2010, it was demonstrated that the genetically engineered lymphocytes expressing chimeric antigen receptors against the CD19 antigen of B Cell could mediate regression of B Cell lymphoma [16]. The above findings using natural and genetically engineered T cells has led to the widespread interest in using adoptive cell therapy in the treatment of human cancers.

The typical method for developing tumor infiltrating lymphocytes involves excising sections of tumor and either dissolving them into single cell suspensions or dividing the excised fragments to be grown in presence of IL-2. This leads to supported proliferation of lymphocytes that kill of the remaining cancer cells in the suspension, and within 2-3 weeks, a pure culture of lymphocytes is obtained [9]. The pure cultures are rigorously tested for anti-tumor activities in assays. Selected cultures are then rapidly proliferated in the presence of irradiated feeder lymphocytes. Within 5-6 weeks of tumor excision, a high concentration of lymphocytes may be obtained for administration into the patients.

An increase in the effectiveness of the therapy is observed when the patients undergo a lympho-depleting routine before the introduction of TILs into the patients. The routine maybe modified for the duration and intensity according to the physician and the patient. In humans, the lymphodepletion regimen induces the release of IL-15 which serves to enhance

the proliferation rate of the infused cell due to a lack of competing endogenous lymphocytes. Lymphodepletion also serves to increase the effect of the infused cell by inducing Toll-Like Receptor (TLR) mediated antigen presentation in APCs [15].

The observation that TILs can mediate regression in melanoma has helped raise interest in the use of the therapy for other tumor histologies.

It has been hypothesized that mutations in cancer cells might be the site of recognition for the TILs as several studies have shown that the target for TILs might be the various nonsynonymous peptides coded by the mutations in melanoma cells. However, the lymphocytes are not capable of recognizing all the mutations as the peptide produced must be able to be excised into sequences of approximately 9 amino acids so that it may be presented by the Major Histocompatibility Complex I while it may be a longer to be used by Major Histocompatibility Complex II. Such peptides maybe identified by the study of peptides with 20-25 amino acids, containing a mutation in the middle flanked by non-mutated residues. Using bioinformatics tools, the binding affinity of these peptides with MHC was tested and those with highest binding affinity may be synthesized and tested under laboratory conditions. Another method involves designing short DNA sequences that may be capable of producing the likes of above mentioned amino acids. The DNA sequence is cloned and transcribed into an RNA sequence which is then electroporated into the antigen presenting cells that might express the peptide. The APC is then tested for binding affinity with MHCs. The lack of an autoimmune response in the case of TIL might too be explained by the mutation target theory [6].

To increase the reach of ACT, genetic engineering has been used to introduce desired T cell receptors in host T Cells as their selectivity can be modified at will. Chimeric antigen receptors (CARs) can be produced by linking the variable regions of light and heavy chains of an antibody with intracellular signaling molecule. Linkage of additional sequences to the CAR might be done to enhance its immune stimulation capabilities. The chimeric sequences are usually transferred into the host cells using lentivirus and gamma retrovirus, however newer techniques such as transposon systems and CRISPR Cas9 are also being experimented with [16]. The selection of appropriate T cell subpopulation and the antigenic targets of the modified cell is of prime importance. Therefore, CARs are artificial receptors that recognize specific antigen present on the surface of the tumor cell and is thus independent of MHC presentation.

On the other hand, the TCR receptors introduced in the cells is composed of an alpha and a beta chain which help recognize the antigen presented by the MHC of the patient.

The acceptance of ACT in the mainstream therapy of cancer depends upon the identification of suitable target molecules for immunologic action. The hunt for monoclonal antibodies for targets expressed only by tumor cells and not normal cells has been going on

for a few decades, but rather unsuccessfully. Several studies to increase the potency of the T cells used in the therapy are underway such as in vitro proliferation of undifferentiated T Cells, improvement in lymphodepletion routine and improvement in vectors, which are likely to improve the clinical viability of ACT in the near future. Adoptive cell therapy, being one of the more risky and expensive therapy has been under public scrutiny, especially as an option for widespread healthcare option, as the personalized nature of the medicine does not suit the mass production tendencies of most pharmaceutical companies. For the introduction into widespread usage, the effectiveness of the procedure must overtake the tedious nature of the therapy.

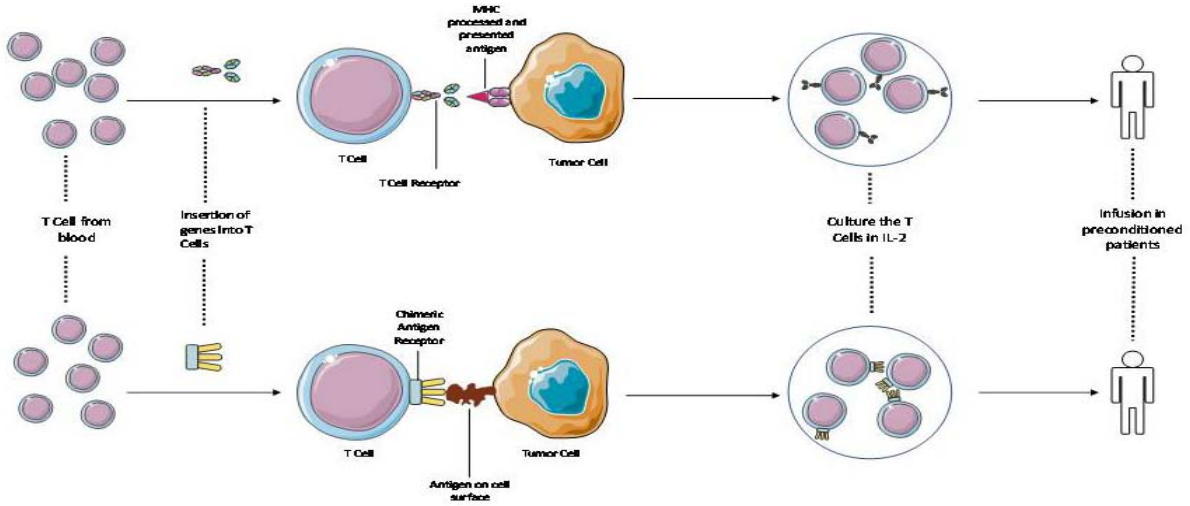

Figure2: Gene modification of lymphocyte

Because of the dual nature of critical factors in the tumor microenvironment, it becomes imperative to study them in even more depth than is currently being done so that we can utilize them as targets for immune therapy. Immunotherapy is a promising approach for the development of integrative therapies for cancer. In combination with strategies such as surgery, chemotherapy and radiation therapy, immunotherapy can provide a tool to efficiently attack residual disease and provide prolonged tumor-specific survival.

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Chapter 2

Mitochondrial Diabetes – An overview

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1. Introduction

The most urgent problem in the field of diabetology, and one of the most important challenges for the XXI century medicine, is to find cure for type 2 diabetes mellitus (T2D). It is estimated that the number of people with diabetes worldwide exceeds 200 million and most of them are T2D patients. In the industrialized world the prevalence of this disease has reached an epidemic proportion and is still growing [1]. The adoption of a sedentary lifestyle, the consumption of non-traditional foods, and a genetic predisposition to the disease are thought to be the major underlying causes of the epidemic. In addition to the worrisome increase in the prevalence of diabetes mellitus (DM), the society at large will be further burdened with problems associated with various macro and microvascular complications of T2D. A major part of this burden (75%) will be borne by developing countries and India will be having the dubious honor of being host to the maximum number of diabetics and it is already called the diabetes capital of the world. Compounding factors like high prevalence of tuberculosis, unfavorable pattern of central obesity and inadequate health facilities add to the difficult survival of diabetics in India [2].

For many decades T2D (non insulin- dependent diabetes), has been regarded a less dangerous type of disease by both the patients and their doctors. But recent estimation revealed T2D as a leading cause of premature death, mainly due to cardiovascular causes and due to occurrence of complications that can lead to blindness, amputations, and renal insufficiency. The life expectancy of millions of patients is shortened due to the diagnosis of T2D [3]. The disease imposes huge economic burden on patients, their families, local communities, health care systems, and societies [4]. Hence T2D was considered as a major medical burden on

society.

Type 2 diabetes is characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both [5]. Interaction of genetic and environmental factors plays a major role in disease incidence. The looming epidemic of T2D is expected to trigger a steep rise in the complications such as ischemic heart disease, stroke, neuropathy, retinopathy and nephropathy. Moreover, there is growing evidence that genetic background also influences the complications of T2D [6-9]. Hence developing better treatments and novel prevention strategies for T2D is a matter of great urgency to provide patients and their families with prognostic advice. To accomplish this goal, it is necessary to understand the pathogenesis of T2D and its complications.

2. Understanding the Genetics of Type 2 Diabetes

Over the last three decades enormous efforts have been undertaken to understand the genetic basis of T2D and defects of beta cell function were recognized increasingly in patients with diabetes [10]. Several genes, such as the insulin gene [11], the insulin receptor gene [12], and the glucokinase gene [13] have been reported to be responsible for the subsets of the disease. These genes encode factors necessary for the metabolic processes from the insulin synthesis and secretion in pancreatic beta cells to the insulin action on various target cells. Apart from these genes, a pivotal role of mitochondria in the pathogenesis of T2D is underlined by the finding that mitochondrial DNA (mtDNA) mutations in humans, as well as deletion of mitochondrial genes in pancreatic beta cell animal models, reduces oxidative phosphorylation (OXPHOS) capacity and causes diabetes [14,15]. Data reported by different investigators suggest that beta cells normally contain a filamentous network of mitochondria, but when mitochondria become chronically fused or fragmented, glucose stimulated insulin secretion (GSIS) is impaired [16-18]. Abnormal mitochondrial morphology and function was observed in pancreatic beta cells from the postmortem studies of T2D patients [19].

The mitochondrial genome of mammalian cells encodes 13 polypeptides, 2 rRNAs and 2 tRNAS. The mitochondrially synthesized polypeptides are constituents of four enzyme complexes involved in OXPHOS and ATP production. Mitochondrial OXPHOS and ATP production in pancreatic beta cells are generally accepted to play a significant role in insulin secretion in response to glucose and other nutrients [20]. This clearly suggests the possible role of mitochondrial defect in GSIS of pancreatic beta cells.

Till now, a number of mtDNA defects have been implicated in the development of diabetes in various populations [21-24]. Most of the studies revealed one or more number of base substitutions in the $tRNA^{Leu}$ gene as the possible causative factor for T2D. As far as the T2D is concerned, genes encoding the mitochondrial respiratory chain play a crucial role in the production of ATP which subsequently releases the secreted insulin once it reaches the

threshold level inside the pancreatic beta cells. But sufficient data is not available to confirm the significant role of the mitochondrial defects in the development of T2D. Even though the history of mitochondria dates back to millions of years, the mitochondrial genetics is just 150 years old as the role of mitochondria in human diseases was realized only in 1962 after the description of a young woman with non-thyroidal hyper metabolism [25]. The genetics of mitochondrial diseases came to the limelight only in 1988 after the reports of a point mutation in Leber hereditary optic neuropathy (LHON) and large-scale deletions in mitochondrial myopathies [26].

Hence molecular basis of the mitochondrial diabetes needs extensive investigation to identify the location/region responsible for disease development. Mitochondrial DNA biology is also found to be complex in nature, however all the pathogenic mutations can occur at almost any site throughout the mitochondrial genome; hence comprehensive screening requires analysis of the entire mtDNA molecule. Also, nonfunctional homoplasmic variants are common and must be distinguished from functional heteroplasmic defects. Finally, mutations may be missed because of variable tissue expression. This is because the level of the mutated mtDNA in relation to the wild-type mtDNA (% heteroplasmy) varies between tissues, being high in post mitotic tissues, such as skeletal muscle and brain, and low in rapidly dividing tissues, such as blood leukocytes [27]. Hence post mitotic tissue will be the suitable sample for detecting mtDNA mutations than leukocyte DNA, where the occurrence of novel mtDNA mutations level will be very low and go undetected. As a consequence, lead to an underestimation of the true prevalence of mtDNA defects in conditions such as diabetes. But most of the studies concentrated mutations in the blood DNA since it is difficult to get post mitotic tissues. Also the reports on the association of mt DNA defects for the mitochondrial associated diseases through the sequencing of complete mitochondrial genome is less when compared to nuclear genome [23, 28-31].

3. Mitochondrial DNA Mutations and Diseases

The mitochondrial genome has a very high mutation rate, 10- to 17-fold higher than that observed in nuclear DNA. Although mtDNA repair systems do exist [32], they are not sufficient to counteract the oxidative damage sustained by the mitochondrial genome due to its proximity to the respiratory chain complexes in the inner membrane and the ROS they generate. Protective histones are also lacking, thus leading mtDNA more susceptible to mutations.

Number of pathological mtDNA mutations has been known for over a decade, yet their mechanistic is not well understood. The first pathogenic mtDNA mutations were identified in 1988 [26,33]. Since then, over 250 pathogenic mtDNA mutations (point mutations and rearrangements) have been characterized [34], shown to cause a wide variety of diseases with

a heterogeneity of phenotypes and a variable age of onset [35- 42]. The pathogenic mutations has been classified into three broad categories based on its position at mitochondrial region which include (i) point mutations affecting protein-coding genes (oxidative phosphorylation); (ii) point mutations affecting the protein synthetic apparatus; and (iii) large deletions [43].

3.1 Clinical Features of Human mtDNA Disease

A striking feature of mtDNA diseases is their clinical heterogeneity and the presence of heteroplasmy. The fraction of mutant mtDNA may vary from less than 1 % to more than 95 % in affected tissues of patients with mitochondrial disease. In addition, the amount of heteroplasmy varies from tissue to tissue and even between cells within a tissue [44], and, in some cases, heteroplasmy can change also with time [45]. The most functionally drastic mutations are always found in heteroplasmic state, since homoplasmy entails lethality. On the contrary, at modest levels of heteroplasmy even drastic mutations can have a subtle phenotypic effect. Conversely, functionally mild mutations that can segregate to homoplasmy in the germ line without compromising early development might have a profound effect in some specific tissues [43]. Nevertheless, for some mitochondrial diseases the phenotype is independent of mutant mtDNA abundance, suggesting the involvement of other factors. The threshold effect, the age and the environment can also influence the pathogenesis of mitochondrial disorders. In addition, the modulating effect of other mitochondrial and/or nuclear genes could also contribute to the diversity of clinical phenotypes [46]. Because the vast majority of the mitochondrial proteins are nucleus-encoded and correct structure and function of the respiratory chain requires many steps which are under control. Hereditary defects in the complex machinery of transport of nDNA-encoded proteins from the cytoplasm into mitochondria, can cause mitochondrial diseases, although only relatively few such disorders have been documented.

Despite the clinical importance of mitochondrial diseases and the fact that the sequence, the genes and the presumed function of mitochondrial chromosome have been completely described for decades, the molecular mechanisms leading from genotype to clinical phenotype remain unsolved. The pathophysiology of mitochondrial diseases is also not well known. While disruption of OXPHOS is central to mitochondrial diseases, many other factors such as calcium dyshomeostasis, increased oxidative stress, and defective turnover of mitochondrial proteins may also contribute.

3.2. Mitochondrial DNA Genotype-Clinical Phenotype Correlation

It seems to make sense that different mtDNA mutations can cause similar clinical manifestations since they cause disease through defective OXPHOS function. In contrast the same mtDNA mutations was found to cause different disease severity, totally different diseases or even does not cause diseases at al. For example, patients with *Kearns–Sayre syndrome (KSS)*, *Chronic progressive external ophthalmoplegia (CPEO) or Pearson syndrome (PS)*

can all carry the same species of large-scale mtDNA deletions. A3243G mutation, the most common mutation associated with mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) and also found in patients with DM, diabetes with deafness, maternal inherited CPEO and mitochondrial myopathy. Conversely other mutations in tRNA genes or protein coding genes are also implicated in MELAS [47].

The diversity of clinical phenotypes mtDNA can be partly ascribed to the difference between level of heteroplasmy in each patient, between each tissue in same patient or even between the each cell in same tissue. The interactions between the differences in the level of heteroplasmy and tissue or mutation specific threshold can give rise to varied clinical phenotype seen in patients. Several lines of evidence suggest that mtDNA backgrounds, nuclear gene backgrounds as well as environmental factors could be the factors modifying the effect pathogenic mtDNA mutations [48].

3.3. Treatment Strategy

At initial stage, T2D is usually treated with a single oral agent. Consistent with the progressive nature of the disease, patients often eventually treated with one or more additional oral agents and in many cases insulin [49,50]. Choice of specific agents is based on individual patient circumstances, including the need for weight loss and control of fasting versus postprandial glucose, the presence of dyslipidemia and HT, and the risk for and potential consequences of hypoglycemia [51]. Type 2 diabetes patients with severely uncontrolled and symptomatic hyperglycemia are best treated, at least initially, with a combination of insulin therapy and lifestyle intervention, often with metformin.

3.3.1. Antihyperglycemic Treatment Strategies

Lifestyle measures, medical nutrition therapy and appropriately prescribed physical activity were recommended for almost all patients with T2D, as well as weight loss for those who are overweight or obese. Unfortunately, many patients were failed to achieve glycemic goals with lifestyle measures alone and required the addition of pharmacotherapy [52]. Extensive development of new therapies during the past 15 years has resulted in more than 11 classes of approved antihyperglycemic medications with diverse mechanisms of action and varied effects on Hb_{A1c}, body weight, lipids, and other factors [53, 54]. These includes Sulfonylurea, Biguanides, Alpha-glucosidase inhibitors, Thiazolidinediones (TZD), Meglitinide, Dipeptidyl peptidase (DPP)-4 inhibitor, Bile acid sequestrant, Sulfonylurea and biguanide, Biguanide and glitazone, Sulfonylurea and glitazone, Biguanide and DPP-4 inhibitor.

3.3.2. Incretin-Based Therapies

Incretin-based therapies are currently part of the antihyperglycemic armamentarium

for the patients with T2D [53, 55]. These include GLP-1 receptor agonist exenatide and the DPP-4 inhibitors sitagliptin and axagliptin. The most recent update of the consensus algorithm statement of a joint ADA/EASD writing group included GLP-1 receptor agonists (but not DPP-4 inhibitors) in tier 2 of preferred agents, especially for patients who have concerns related to weight and hypoglycemia [51]. They noted that DPP-4 inhibitors may be appropriate choices in selected patients.

3.3.3 Antioxidant Therapy

Apart from these antihyperglycemic agents, additionally T2D patients have to be prescribed with antioxidants to limit mitochondrial radical production during hyperglycemia and to counteract their damaging effects. This may be useful complements to normalize blood glucose, as well as protecting peripheral tissues from hyperglycemia-induced oxidative damage. Antioxidants may have the additional benefit of improving GSIS, both by preventing the damage to β-cells and possibly by blocking the proposed ROS activation of UCP2 in β -cells. The advantage of natural antioxidants is their safety and that large oral doses are well tolerated [56]. To date, mitochondria-targeted versions of Coenzyme Q and vitamin E have been made and can be administered safely to mice [57].

Coenzyme Q_{10} administration to GK rats showed no success in preventing mitochondrial dysfunction [58]. The ineffectiveness of currently existing antioxidants in ameliorating oxidative-stress-mediated diseases points to the need in developing mitochondria-targeted antioxidants. Triphenyl phosphonium-based, amino-acid and peptide-based antioxidants have been shown to protect mitochondria against oxidative insult, which indicates mitochondrially targeted antioxidants are future promises for disease treatment.

3.2. Therapies in Development

Incretin-based therapies are currently in development which includes a novel once-weekly formulation of exenatide; taspoglutide, another once-weekly glucagon-like peptide (GLP) -1 receptor agonist; and liraglutide, a GLP-1 receptor agonist that is administered once daily (59). Liraglutide is currently being evaluated in clinical trials as a once-daily subcutaneous injection. Liraglutide has been reported to reduce Hb_{A1c} by 1.1 % at 26 weeks and up to 1.14 % at 52 weeks and result in weight loss (up to 2.8 kg at 26 weeks and up to 2.5 kg at 52 weeks) in patients with T2D who are treatment- naive or taking other antidiabetes agents, including metformin, sulfonylurea, and TZD (60-62). Evaluation of the once-weekly formulation of exenatide showed reductions in Hb_{A1c} of 1.9 % at 30 weeks and 2.0 % at 52 weeks with a weight loss of 3.7 kg at 30 weeks and 4.1 kg over 52 weeks of treatment [63,64].

4. Summary

Mitochondria play a primary role in the etiology of genetic forms of "mitochondrial" diabetes. Mitochondrial ATP plays a crucial role in the regulation of insulin release from the pancreatic β-cells. When the production ROS exceeds the threshold level, the capacity of β-islets in secreting insulin deteriorates gradually particularly in type 2 diabetes. This in turn leads to the patient to develop multiple complications such as coronary artery disease, neuropathy, retinopathy, nephropathy etc. Currently available treatment such as *Glimepiride, glimepiride-pioglitazone, glimeperide-rosiglitazone, gliclazide. glipizide glipizide-metformin, glyburide, glyburide-metformin etc* does control the level of glucose in the blood, however, there is no treatment which address both mitochondrial function and ROS production. Hence, new treatment strategies regulating mitochondrial biogenesis, ROS and respiration would help the diabetes patients in future.

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Advances in Biotechnology

Chapter 3

Microalgae: A Potential Candidate for Biodiesel

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Abstract

Modern way of life intimately depends on fuels that are derived from fossil resources. With the depletion of resources and to meet the demand of the diesel fuel industry, alternative oil sources are being explored and developed in recent days. Biofuels derived from renewable biomass, organic matter could minimize the use and reduce the dependency on fossil fuel. It is eco-friendly, non-toxic, bio-degradable, stable, reduces the level of potential or probable carcinogens and has a favourable emission profile. Oleaginous microorganisms such as fungi and microalgae with 20% or more lipids in their cell have emerged as a potential feedstock for biodiesel production. Microalgal biodiesel production is considered to reduce the overall production costs of biodiesel in the global market, which is the major reason for researchers focusing their attention on oleaginous microalgae. Of late, combinatorial approaches such as genetic engineering and molecular engineering have been implemented in order to develop efficient microalgal platforms for the production of biodiesel. The present chapter describes the rapid progress made in this area in the past ten years.

1. Introduction

High energy prices, global warming, burgeoning population and uncontrolled urbanization are drawing considerable attention to find a renewable biofuels. The basic sources of energy are fossil fuels- petroleum, diesel, natural gas, coal and nuclear energy. Over 1.5 trillion barrels

of oil have been produced since Edwin Drake drilled the world's first oil well in 1859 [1]. It is estimated that, the same amount is required to meet the global demand in the next 25 years alone. In 2008, the annual world primary energy consumption was estimated as 11,295 million tonnes of oil equivalent. Fossil fuels accounted for 88% of the primary energy consumption, with oil (35%), coal (29%) and natural gas (24%) as the major fuels, while nuclear energy and hydroelectricity account for 5% and 6% of the total primary energy consumption respectively. It is estimated that the global demand for petroleum will be increased to 40% by 2025 [2].

Extensive use of fossil fuels for transport, electricity and thermal energy generation has led to the emission of greenhouse gases (GHGs) to the atmosphere, thus contributing to global warming. They account for 98% of total carbon emission [3]. Combustion of fossil fuels emits more than 6 billion tonnes of carbon-di-oxide annually in the atmosphere (Fig.1) In 2006, associated GHGs emissions were 29G tonnes [4]. It is estimated that natural processes confiscate only about 12G tonnes. Petroleum diesel combustion also contributes for green house emissions. Furthermore, it is also a major source of other air contaminants including nitric oxide, sulphur oxide, carbon monoxide, particulate matter, carcinogens and volatile organic compounds. Therefore, it is important to develop suitable strategies and stringent policies to minimise the impact of excess GHGs [5]. Another disadvantage with petroleum based fuels is their uneven distribution in the world (Fig.2), followed by decline in its reservoirs (at a rate of 2-3% predicted per year starting in 2010 [6].

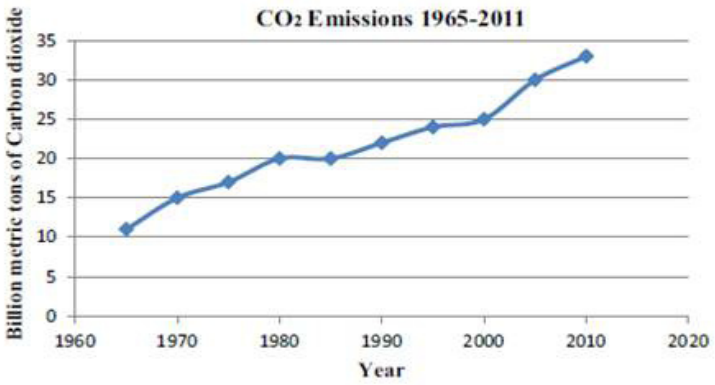

Figure 1: Graph showing global increase in carbon-di-oxide emission

Source: [7]

Of late, with the rapid increase in the price of crude oil and projected decrease in fossil fuel and petroleum reserves, followed by the growing concern of the environmental hazards of the non-renewable fuels has stimulated researchers to quest for alternative, sustainable and renewable energy sources.

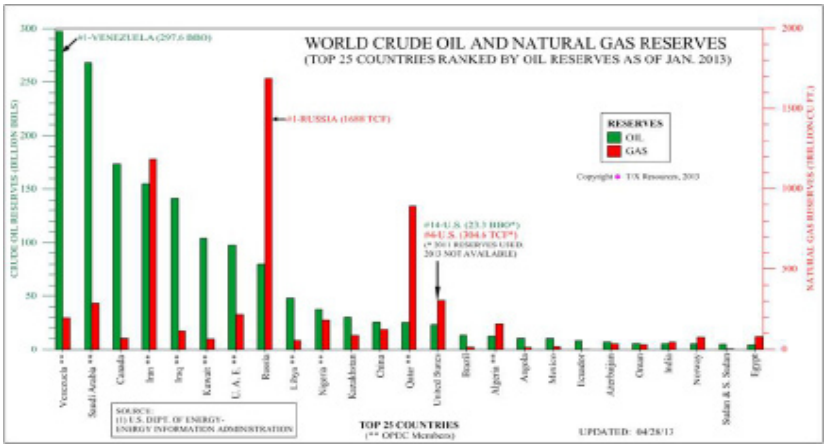

Figure 2: Global distribution of crude oil and natural reserves

Source: [6]

1.1. Development of biofuel resources

Finding suitable auxiliary clean energy for the future is one of the society's most daunting challenges and is associated with global stability, economic prosperity and quality of life. Of late, production of biofuels from renewable resources such as plants or organic waste, oleaginous micro-organisms has received considerable attention. It is eco-friendly, biodegradable and sustainable renewable resources.

1.2. Classification of biofuels

Biofuels are classified as primary and secondary biofuels. Primary biofuels are used in crude form, primarily for heating, cooking or electricity production such as fuel wood and wood chips etc. Whereas, secondary biofuels are produced by biomass processing (e.g. bioethanol, biodiesel etc). It can be blended with petrol to drive the vehicles and in various industrial practices. Secondary biofuels are further divided as first, second and third-generation biofuels on the basis of raw material and the technology used for their production (Fig.3). Biofuels can be solid, such as fuel wood, charcoal, and wood pellets, in liquid form such as ethanol, butanol and biodiesel and gaseous such as biogas (methane) [8].

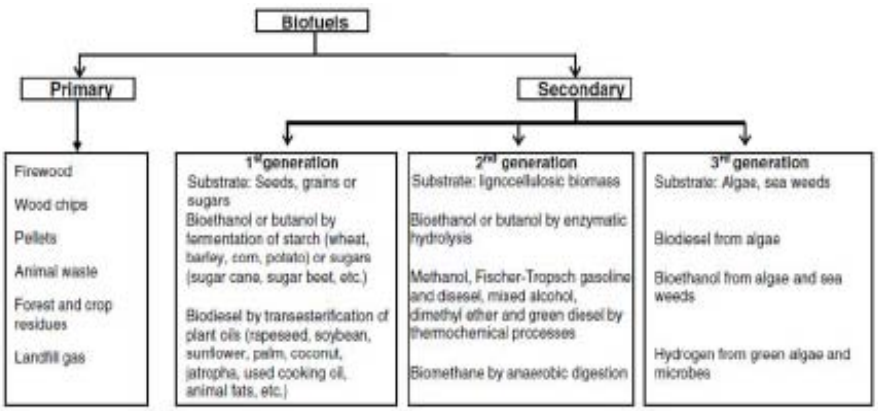

Figure 3: Classification of biofuels

Source: [8]

1.2.1 First Generation Biofuels

First generation biofuels such as bioethanol is the most promising alternative renewable energy source and has attained commercial level production in several countries like Brazil and United States Of America [9].Together, these countries account for 89% of the current global bioethanol production [10]. It is a liquid fuel produced by fermenting sugar extracted from lignocellulose [10], corn starch [11], sugarcane bagasse [12], sugar beets [13] and molasses [14], grains or seeds [15, 16]. It improves fuel combustion in vehicles, thereby, reducing GHGs. In Brazil, bioethanol accounts for 40% fuel needs [17].

Biodiesel, a monoalkyl esters of long chain fatty acids with short chain alcohols, primarily methanol and ethanol, resulting in fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs) [18]. It is obtained from dedicated oleaginous plants such as pongamia, jatropa etc by transesterification processes. It is eco-friendly, biodegradable, stable, reduces GHGs emission, low flammability and good lubrication properties [19]. Pure biodiesel or biodiesel blended in any ratio with petroleum-based diesel can be used in conventional diesel engines with no or only marginal modifications.

However, the first generation biofuels seemed to create scepticism to scientists. As vegetable oil is used for human consumption, harnessing it for biodiesel production could lead to an increase in price of food-grade oils. The extensive plantation of oil yielding plants could lead to land competition and biodiversity loss [20]. The cost of biodiesel production mainly depends on the price of the feedstocks that accounts for 60-75% of the total cost of biodiesel production [21]. To become a potential alternative fuel, biodiesel must compete economically with diesel.

1.2.2 Second Generation Biofuels

Transition to second generation biofuels has attracted great attention. It is produced from two methods i.e. biochemical or thermochemical processing from agricultural ligno-cellulosic biomass (non-edible crop residues or whole plant biomass) and industrial or municipal organic waste. It is eco-friendly, inexpensive, renewable, reduces land requirement and limits the direct food versus fuel competition [22]. Biomass conversion by thermochemical method is achieved at extreme temperatures and pressures. The fuel thus obtained can be used directly in engines. Whereas, biochemical conversions, also called as saccharification involves application of array of enzymes such as cellulase, amylase, β-glucosidases, xylanase [23] obtained from fungi [24] and bacteria [23] on residual substrates such as ligno-cellulosic biomass [25], rice straw [26], sugarcane bagasse [27], molasses [28], sugar beet pulp [29] and starch [30]. However, it is cost effective, requires sophisticated equipment and larger-scale facilities which limits its economic feasibility and commercial production [31].

1.2.3 Third Generation Biofuels

Growing lines of evidence suggest that, micro-organisms such as yeast, fungi and microalgae can accumulate large amount of lipid. This has attracted great attention and can be used as potential candidate for third generation biofuel production. Bacteria, in general, do not produce triacylglycerols but instead, accumulate poly-β-hydroxy-butyrates and alkanaoates as storage polymers [32]. Several benefits can be envisioned from yeast, algae and fungi due to their advantages over higher plants such as similarity in fatty acid profiles with plant seed oils, easy to grow, simple cultural conditions and nutrients for growth, no requirement of agricultural land and consistency of the product yield has been shown to be an ideal alternative owing to its amicability for the separation, purification and industrialization [33]. Furthermore, it is devoid of the major drawbacks associated with first and second generation biofuels. Screening the potential oleaginous microbial cell factories or engineered strains for biodiesel production could be a promising way for renewable energy. The manipulation and regulation of microbial lipid biosynthesis opens a new avenue for academic researchers and harness its potential in its commercial application for biodiesel production.

2. Microalgae for Biodiesel Production

Microalgae comprises several groups of unicellular and multicellular, colonial or filamentous, photosynthetic or heterotrophic micro-organisms containing chlorophyll and other pigments. It can grow autotrophically or heterotrophically with a wide range of tolerance to different temperature, salinity, pH and nutrient [34]. More than 40,000 microalgal species have been classified as prokaryotes (cyanobacteria) and eukaryotes such as green algae, diatoms, yellow–green algae, golden algae, red algae, brown algae, dinoflagellates [35,36]

2.1. Classification

Algae is classified into four types

1. Prokaryotic Algae: **Cyanophyta-** Cyanobacteria are the only prokaryotic algae. It consist of chlorophyll and phycobiliproteins.

2. Eukaryotic Algae: It consist of chloroplasts which is surrounded by two membranes of the chloroplast envelope.

a. Phylum Glaucophyta: It includes algae that represent transitional position in the evolution of chloroplasts; photosynthesis is supported by modified endosymbiotic cyanobacteria. Example- *Glaucocystis*

b. Phylum Rhodophyta: It comprises Chloro phyll *a,* phycobiliproteins, flagellated cells are absent, storage product is floridean starch. Example - Red algae

c. Phylum Chlorophyta: It comprises chlorophylls *a* and *b*, storage product is starch. It is found inside the chloroplast. Example: Green algae

3. Eukaryotic algae: It consist of chloroplast which is surrounded by one membrane of chloroplast endoplasmic reticulum.

a. Euglenophyta : It comprises chlorophyll *a* and *b*, one flagellum with a spiral row of fibrillar hairs and proteinaceous pellicle in strips are present under the plasma membrane; storage product is paramylon; character istic type of cell division. Example: Euglenoids

b. Dinophyta (dinoflagellates)): it comprises mesokaryotic nucleus, chlorophyll *a* and *c*. Cell is commonly divided into an epicone and a hypocone by a girdle and helical transverse flagellum.

a. Apicompexa : they are heterotrophic flagellates with colorless plastids.

4. Eukaryotic algae with chloroplasts are surrounded by two membranes of chloroplast endoplasmic reticulum.

a. Cryptophyta: Nucleomorph present between inner and outer membrane of chloroplast endoplasmic reticulum. Starch is stored in the form of grains between inner membrane of chloroplast endoplasmic reticulum and chloroplast envelope. It consist of chlorophyll *a* and *c*, phycobiliproteins are present. Periplast are seen inside the plasma membrane. Example : Cryptophytes

b. Heterokontophyta : It usually consist of anterior tinsel and posterior whiplash flagellum. It consist of chlorophyll *a* and *c* along with fucoxanthin. Storage product is in the form of chrysolaminarin, present in the heterokonts.

Example : *Paraphysomonas sigillifera*.

Heterokontophyta consist of the following classes Chrysophyceaeec, Synurophyceae, Eustigmatophyceae, Pinguiophyceae, Dictyochophyceae, Pelagophyceae, Bolidophyceae, Bacillariophyceae, Raphidophyceae, Xanthophyceae, Phaeothamniophyceae, Phaeophyceae, Prymnesiophyta.

2.2 Selection and screening of oleaginous microalgae for biodiesel production

Due to variation and diversity of microalgal lipids in nature, selection of oleaginous microalgal strains suitable for biodiesel production requires screening of large number of

microalgal strains. In 1978, the first large-scale collection and screening of oleaginous algae was started, when the Aquatic Species Program, launched by U.S. National Renewable Energy Laboratory. Over 3000 strains were collected and eventually around 300 species were identified as oleaginous algae [37]. Screening of oleaginous microalgae and optimizing culture conditions to enhance lipid accumulation and evaluation of its potential for biodiesel production is well studied [38, 39, 40, 41]. Screening of microalgae encompass the following steps [Figure 4].

1. Sampling from the field i.e. isolation or collection from algal collection library
2. Identification and maintenance of the culture
3. Biomass harvesting
4. Determination of lipid content oil extraction [42, 43, 44, 45]

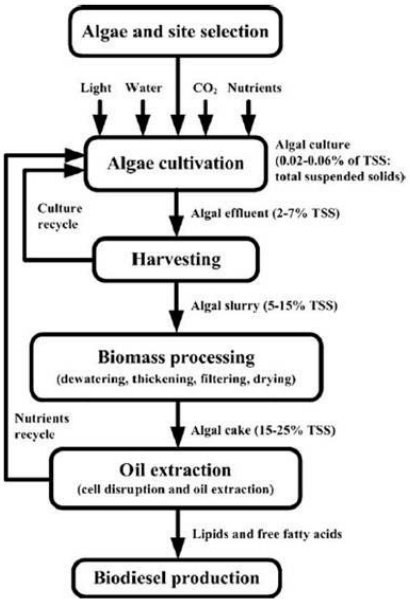

Figure 4: Process of biodiesel production in microalgae

Source: [43]

2.3. Harnessing microalgae for biodiesel production

The advantages of microalgae as an alternate source for biodiesel production over high plants are as follows

1. Rapid growth, accumulates high content of lipid
2. Non-requirement of arable land for its growth
3. Phototropic microalgae marks it to be economical than oleaginous heterotrophic microorganisms that utilize glucose and other organic carbon sources [46]
4. It utilizes large amounts of carbon-di-oxide emitted by power plants and other industrial

sources, thereby contributing to GHG mitigation [36]

5. It also produces other types of biofuels such as alkanes, ethanol, butanol and hydrogen [47]

6. Production of biodiesel from microalgae results in minimal release of sulphur dioxide, nitrous oxide and other contaminants when compared to petroleum-derived diesel [48,42].

2.4. Biochemistry of lipid accumulation in microalgae

The process of lipid accumulation in microbial cells is well documented [49]. Microorganisms in general, are able to synthesize lipids for essential functioning of their membrane structures. However, a few microbes in the microbial kingdom have the ability to accumulate more than 20% lipids in their cells. These are called as oleaginous organisms and they store lipid in oil vacuoles as triacylglycerol. The process of lipid accumulation is known as lipogenesis. The pattern of lipid accumulation and fatty acid profile in microalgal species varies significantly **(Table 1)**. It is influenced by factors such as light intensity [50], nitrogen concentration [51,52], carbon-di-oxide concentration [53], salinity [54], temperature [35], pH [39] etc. Overview of the metabolites and representative pathways in microalgal lipid biosynthesis is depicted in Figure.5.

Table 1: Lipid content in selected microalgae

Marine and freshwater microalgae species	lipid content (% dry weight biomass)	Lipid productivity (mg/L./clay)	Volumetrk productivity of biomass (g/L/day)	Areal productivity of biomass (g/&/clay)
Ankistrodesmus sp.	24.0-31.0	-	-	113-17.4
Botryococcus braunii	25.0-75.0	-	0.02	3.0
Chaetoceros muelleri	33.6	21.8	0.07	-
Chaetoceros cakitrans	14.6-16.4/39.8	17.6	0.04	-
Morella emersonii	25.0-63.0	10.3-50.0	0.036-0.041	0.91-0.97
Chlorella protothecoides	14.6-57.8	1214	2.00-7.70	-
Chlorella sorokiniano	19.0-22.0	44.7	0.23-1.47	-
Chlorella vulgaris	5.0-58.0	11.2-40.0	0.02-020	0.57-0.95
Chlorella sp.	10.0-48.0	42.1	0.02-2.5	1.61-16.47/25
Chlorelia pyrenoidosa	2.0	-	2.90-3.64	72.5/130
Moreno	18.0-57.0	18.7	-	3.50-13.90
Chlorococcum sp.	19.3	53.7	0.28	-
Crypthecodinium cohnii	20.0-51.1	-	10	-
Dunaliella sauna	6.0-25.0	116.0	0.22-0.34	1.6-3.5/20-38
Dunaliella primotecta	23.1	-	0.09	14
Dunaliella tertioleao	16.7-71.0	-	0.12	-
Dunaliella sp.	17.5-67.0	33.5	-	-

Ellipsoidion sp.	27.4	47.3	0.17	
Euglena gracilis	14.0-20.0	-	7.70	
Haematococcus pluviatis	25.0	-	0.05-0.06	10.2-36.4
Isochrysis galbana	7.0-40.0	-	0.32-1.60	
hoc/trysts sp.	7.1-33	37.8	0.08-0.17	-
Monodus subterraneus	16.0	30.4	0.19	-

Source: [48, 45, 60]

Lipids are classified into phospholipids, spingolipids and neutral lipids. Triacylglycerols, main constituents of biodiesel are packed in neutral lipids. Biosynthesis of triglycerides in microalgae may consist of the following three steps:

(a) Formation of acetyl coenzyme A (acetyl-coA) in the cytoplasm

(b) Elongation and desaturation of hydrocarbon chain

(c) Synthesis of triglycerides

(a) Formation of acetyl coenzyme A (acetyl-coA) in the cytoplasm

Microalgae, in the presence of photon energy fix the carbon-di-oxide into sugars. Acetyl-coA is formed during the light reaction and Calvin cycle. It is synthesized in the chloroplast [55]. Further, the 3-PGA is exported to cytoplasm for consumption. Subsequently, carbon is directed for glucose synthesis via glycolysis and is further converted into starch, which acts as a storage product in cells [56].

After Calvin cycle, 3-phosphoglycerate (3-PGA) is synthesized in the chloroplasts followed by the glycolytic pathway to form pyruvate (Fig.5). Pyruvate releases CO_2, generates acetyl-CoA (acetyl coenzyme) in the presence of pyruvate dehydrogenase (PDH). Acetyl-CoA serves as the precursor for fatty acid synthesis in the chloroplast [55].

(b) Elongation and desaturation of carbon chain of fatty acids

In most of the organisms, the elongation of carbon chain of fatty acids is achieved by two enzyme machineries namely acetyl-coA carboxylase enzyme [ACCase] and fatty acid synthase [49]. During fatty acid synthesis, acetyl-coA acts as a primer and malonyl-coA serves as a substrate. Fatty acid synthesis is initiated by ACCase enzyme, it synthesizes malonyl-CoA from acetyl-CoA and bicarbonate. Malonyl-CoA group is transferred to malonyl-ACP (acetyl carrier protein) catalyzed by an acyl carrier protein malonyltransferase. The C16 and C18 fatty acid thio-ester is formed after a series of elongation reactions [57]. Growing body of evidence suggest that, synthesis of short-chain fatty acids in microalgae is similar to other living organism such as plants, animals, fungi and bacteria [49,57]. Desaturation of carbon

chain of fatty acid occurs from C18 and further elongation of carbon chain occurs thereby leading to the synthesis of long-chain fatty acids which are unusual in normal plant oils (Fig.5). Thus, selection of a potential strain is a crucial step for algal biodiesel production.

Triacylglycerol is synthesized by the sequential acylation of glycerol-3-phosphate (G3P) backbone with three acyl-CoAs catalyzed by the enzyme acyltransferases. Acylation of G3P using glycerol-3-phosphate acyltransferase results in the synthesis of lyso-phosphatidic acid. This is further acylated to phosphatidic acid by (lysophosphatidic acid acyltransferase). Furthermore, phosphatidic acid phosphatase removes the phosphate group from phosphatidic acid to generate DAG (diacylglycerol). The oil synthesis is catalyzed by DGAT (diacylglycerol acyltransferase) from DAG to triacylglycerol [55, 57].

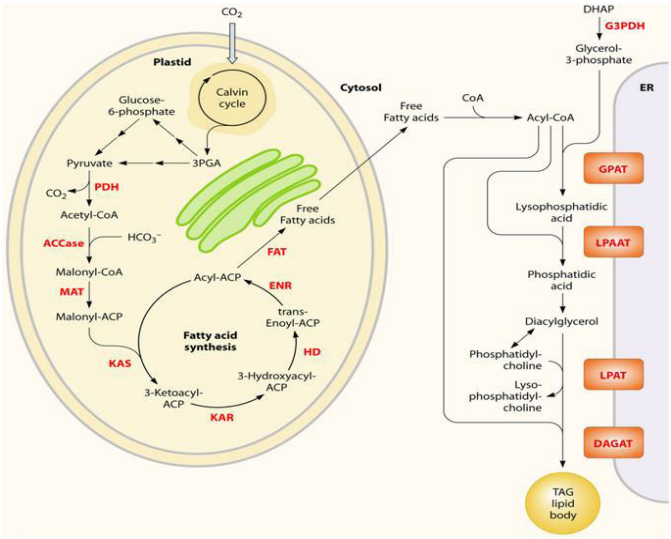

Figure 5: Overview of the metabolites and representative pathways in microalgal lipid biosynthesis shown in black and enzymes in red.

(Free fatty acids are synthesized in the chloroplast, while TAGs may be assembled at the ER. ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; CoA, coenzyme A; DAGAT, diacylglycerol acyltransferase; DHAP, dihydroxyacetone phosphate; ENR, enoyl-ACP reductase; FAT, fatty acyl-ACP thioesterase; G3PDH, glycerol-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; HD, 3-hydroxyacyl- ACP dehydratase; KAR, 3-ketoacyl-ACP reductase; KAS, 3-ketoacyl-ACP synthase; LPAAT, lyso-phosphatidic acid acyltransferase; LPAT, lyso-phosphatidylcholine acyltransferase; MAT, malonyl-CoA:ACP transacylase; PDH, pyruvate dehydrogenase complex; TAG, triacylglycerols).
Source: [43]

2.5. Microalgae lipid accumulation and oil production

Microalgal species can be induced to accumulate substantial quantities of lipids [58, 59] thus contributing to high oil yield. Average lipid content ranges between 1%-70%. However, under certain physiological conditions few species can reach up to 90% of dry weight (Table 1). Although microalgae oil yield is strain-dependent it is generally much higher than other vegetable oil crops **(Table 2, 3** and **4)**.

Table 2: Oil content in selected microalgae

Sl. No	Microalgae	Oil content (wt% of dry basis)
1	*Botryococcus braunii*	25–75
2	*Chlorella sp.*	28-32
3	*Crypthecodinium cohni*	20
4	*Cylindrotheca sp.*	16-37
5	*Dunaliella primolecta*	23
6	*Isochrysis sp.*	25-33
7	*Monallanthus salina*	>20
8	*Nannochloris sp.*	20-35
9	*Nannochloropsis sp.*	31-68
10	*Neochloris oleoabundans*	35-54
11	*Nitzschia sp.*	45-47
12	*Phaeodactylum tricornutum*	20-30
13	*Schizochytrium sp.*	50-77
14	*Tetraselmis sueica*	15-23

Source: [61]

Table 3: Comparison of microalgae with other biodiesel feedstocks

Sl. No	Plant source	Seed oil content (% oil by wt in biomass)	Oil yield (L oil/ha year	Land use (m2 year/kg biodiesel)	Biodiesel productivity (kg biodiesel/ ha year)
1	Corn/Maize (Zea mays L.)	44	172	66	152
2	Hemp (*Cannabis sativa L.*)	33	363	31	321
3	Soybean (*Glycine max L.*)	18	636	18	562
4	Jatropha (*Jatropha curcas L.*)	28	741	15	656
5	Camelina (*Camelina sativa L.*)	42	915	12	809
6	Canola/Rapeseed (*Brassica napus L.)*	41	974	12	862
7	Sunflower (*Helianthus annuus L.)*	40	1070	11	946
8	Castor (*Ricinus communis*)	48	1307	9	1156
9	Palm oil (*Elaeis guineensis*)	36	5366	2	4747
10	Microalgae (low oil content)	30	58,700	0.2	51,927
11	Microalgae (medium oil content)	50	97,800	0.1	86,515
12	Microalgae (high oil content)	70	1,36,900	0.1	1,21,104

Source: [43]

Table 4: Yield of various plant oils

Sl. No	Crop	Oil in litres per hectare
1	Algae	1,00,000
2	Castor	1413
3	Coconut	2689
4	Palm	5950
5	Safflower	779
6	Soy	446
7	Sunflower	952

Source: [61]

3. Properties of biodiesel

Physicochemical properties of microalgal biodiesel are nearly similar to diesel fuel. Important properties of biodiesel are cetane number, heat of combustion, viscosity, oxidative stability, cold flow properties and lubricity [62]. The main properties of microalgal biodiesel compared with diesel and first generation biodiesel is shown in Table 5.

3.1. Cetane number

It determines the quality of ignition of a fuel which increases with the number of carbon and decreases with the number of unsaturated carbon bounds [63]. A higher unsaturated biodiesel like microalgae biodiesel would have a lower cetane number.

3.2. Heat of combustion

It indicates if a biodiesel is suitable to burn in a diesel engine. The heat of combustion increases with the length of the carbon chain [64]. In 2004, Miao and Wu reported that, lipids extracted from heterotrophic microalgae in the presence of sulphuric acid in methanol, obtained a biodiesel with a heat of combustion of 35.4 MJ/L which is in the range of diesel fuel (36-38 MJ/L) [65].

3.3. Viscosity

It increases with the number of carbon and decreases with the degree of unsaturation. A higher kinematic viscosity would create engine problems like engine deposits [64]. Transesterification decreases the viscosity of the oil at values usually between 4 to 6 mm/s (40°C) [66].

3.4. Oxidative stability

When fatty acid methyl esters (FAME) reacts with oxygen, hydrogen peroxides, aldehydes, acids and other oxygenates are formed, which could deposit in the engine [64]. It

entirely depends on the degree of unsaturation [63]. Oxidation stability of microalgal lipids is therefore a real problem [67]. It can be overcome by adding antioxidants if the biodiesel blend is stored more than a few months [66].

3.5. Cold flow properties

It is an important parameter for biodiesel production in European countries such as Canada. Decrease in temperature could lead to the formation of visible crystals in the biodiesel at a limit called as cloud point [64]. Cloud point temperature decreases with the mole fraction of unsaturated compounds and slightly increases with the length of the carbon chain [68].

3.6. Lubricity

Lubricity for a fuel is "the ability to reduce friction between solid surfaces in relative motion" [69]. The lubricant of diesel fuel is influenced by the viscosity, acidity, water content and the sulphur compounds [70]. For microalgae biodiesel, no lubricant study is yet reported from the literature.

Table 5: Comparison of properties of microalgal oil, conventional diesel fuel, and ASTM biodiesel standard

Sl. No	Properties	Biodiesel from microalgal oil	Diesel fuel	ASTM biodiesel standard
1	Density (Kg/l)	0.864	0.838	0.84-0.90
2	Viscosity (mm^2/s, cSt at 40°C	5.2	1.9-4.1	3.5-5.0
3	Flash Point (°C)	115	75	Min 100
4	Solidifying Point (°C)	-12	-50 to 10	-
5	Cold filter plugging point (°C)	-11	-3.0 (max -6.7)	Summer max 0; winter max <-15
6	Acid value (mg KOH/g)	0.374	Max 0.5	Max 0.5
7	Heating Value (MJ/Kg)	41	40-45	-
8	H/C ratio	1.81	1.81	-

Source : [71]

4. Mass Cultivation of Microalgae

Large-scale production of microalgal biomass generally uses continuous culture system during daylight. In this method, fresh algal culture medium is fed at a constant rate and the same quantity of microalgal broth is withdrawn continuously. However, feeding ceases during the night, but the mixing of the culture medium should continue to avoid flustering of the biomass [72]. As much as 25% of the biomass produced during daylight, may be lost during the night because of respiration. The extent of this loss depends on intensity of sunlight under which the biomass was grown, temperature during day and night time. In general, for large-scale production of microalgae, raceway ponds [3, 73] and tubular photobioreactors [3, 74] are widely used.

4.1 Open Pond System

It is also known as "Raceway Pond System". At present, about 98% of commercial algae are cultivated using this system [75]. It is made up of a closed loop recirculation channel which is 0.3m deep (Fig.6). Mixing and circulation is mechanically achieved by paddlewheels, which are limited to 20cm- 30cm in depth (Fig.6). Flow is directed around bends by baffles placed in the flow channel. They are constructed from concrete, however, compact earth-line ponds lined with plastic have also been used [3]. During daylight, the culture is fed continuously in front of the paddlewheel where the flow begins (Fig.6). On completion of the circulation loop, broth is harvested behind the paddlewheel, which is operated continuously to prevent sedimentation.

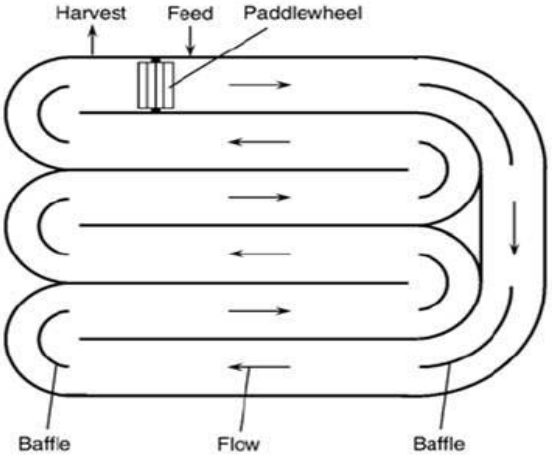

Figure 6: Aerial view of raceway pond

Source [45]

Raceway ponds is most suitable for mass cultivation of microalgal species which can tolerate high salinity and pH such as *Dunaliella salina, Spirulina, Chlorella species* etc [76]. Microbial contamination, seasonal variation and temperature fluctuations directly impede the biomass production [77, 45]. Due to low productivities, large areas of land may be required to meet the desired output of cultivation [76]. Maintenance and cleaning of open ponds are easier and less energy intensive than photobioreactors [3]. Although raceways are economical, they have a low biomass productivity compared with photobioreactor [77, 3].

4.2. Photobioreactor

Photobioreactors (PBRs) have received much attention because of its versatility, high biomass productivity and ease to control culture conditions [78,79]. Various types of photobioreactors used in microalgal mass cultivation are horizontal tubular PBRs, stirred PBRs, airlift and bubble column photobioreactor [79,80,81]. They are more versatile than open ponds as they can use sunlight, artificial light and various combinations and intensities of light sources. Advantages and disadvantages of the respective PBRs are summarized in Table. 6

Tubular PBRs is commonly used for mass cultivation of microalgae [81]. The productivities of PBRs are influenced by the light supply, carbon-di-oxide and fluctuations in temperature, pH, and dissolved oxygen levels [82]. It consist of a series of straight, transparent solar tubes which allows the light to pass through the dense culture **(Fig.7)**. It is made up of plastic or glass with 0.1m in diameter. The orientation of the solar collector may be horizontal, vertical, inclined or as a helical coil around a supporting frame [3,79]. Microalgal broth is circulated from a reservoir (i.e. the degassing column in **Fig.7**) to the solar collector and back to the reservoir. The solar tubes are placed parallel to each other and flat above the ground (Figure.7). Horizontal, parallel straight tubes are sometimes arranged like a fence (Figure. 7). The tubes are always oriented North–South direction **(Fig.7)**.

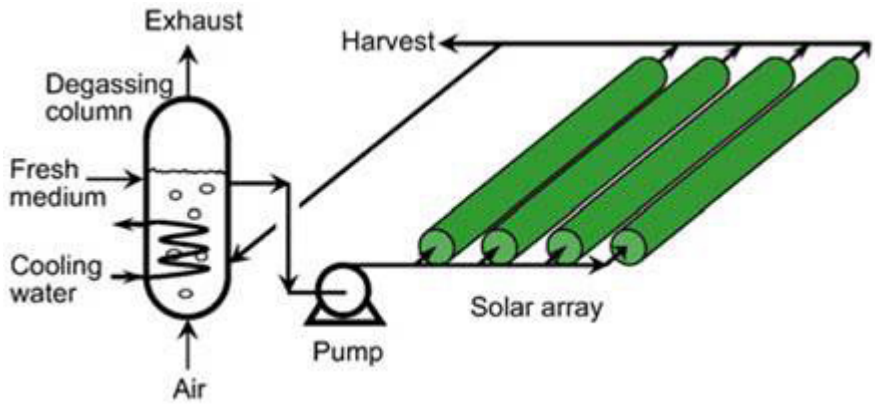

Figure 7: A tubular photobioreactor

Source: [45]

Photosynthesis generates oxygen. Therefore, cultures are generally reticulated by pump passing through a degasser at regular intervals in order to remove excess oxygen (Fig .7). Higher levels of oxygen lead to lower productivities due to photo-oxidative stress. As the broth moves along a photobioreactor tube, pH increases because of consumption of carbon dioxide [83]. Additional carbon dioxide injection points is required to prevent carbon limitation and an excessive rise in pH [72]. As much as 25% of the biomass produced during day light could be consumed during the night to sustain the cells until sunrise. However, this problem can be overcome by lowering the temperature at night.

Table 6: Advantages and disadvantages of different Photobioreactors

Sl. No	Type of photobioreactor	Advantages	Disadvantages
1	Horizontal tubular PBR	High light conversion efficiency	a. Causes photo bleaching due to high concentration of dissolved oxygen and thus reduces photosynthesis efficiency
2	Strirred PBR	a. Expedient b. Carbon-di-oxide can be supplied efficiently	a. Lack of internal light b. Mechanical agitation limits its use c. Low surface area
3	Airlift PBR	a. High biomass production b. uniform mixing can be achieved c. low hydrodynamic stress d. Best suitable for immobilization of algae	Cost- effective
4	Bubble column PBR	a. Economical b. Efficient release of oxygen	a. Lack of internal light b. Lack of mixing

4.3. Advantages and limitations of raceway ponds and photo bioreactors

In contrast to open ponds, photobioreactors have the advantages of low contamination, high productivity, minimal evaporation, reduced CO_2 losses and better control over culture conditions (Table.7). The major drawbacks of photobioreactors are the high costs of construction, fluctuations in temperature [85], pH [84,85], oxygen [86], light [87] and carbon-di-oxide [85]. Although these can be partially compensated by higher productivity, they still limit the cost-effective production of microalgal biomass on a scale required for biodiesel production. Hybrid algae production system comprising photobioreactors and open ponds may be a promising way. Sufficient contaminant-free inoculum can be produced in photobioreactors, followed by transfer to open ponds or raceways to attain the biomass needed for biodiesel production [88].

Table 7: Comparison between open ponds and photobioreactor

Sl. No	Culture systems for microalgae	Open Ponds	Photobioreactors
1	Contamination control	Difficult	Easy
2	Contamination	High	Low
3	Energy consumption	Low	High
4	Process control	Difficult	Easy
5	Species control	Difficult	Easy
6	Mixing	Very poor	Uniform
7	Operation regime	Batch / semi-continuous	Batch / semi-continuous
8	Space required	More	Less
9	Population (algal cell) Density	Low	High
10	Investment	Low	High
11	Operation costs	Low	High
12	Light utilization Efficiency	Poor	High
13	Temperature control	Difficult	Easy
14	Productivity	Low	3–5 times more productive
15	Hydrodynamic stress on algae	Very low	Low–high
16	Evaporation of growth Medium	High	Low
17	Gas transfer control	Low	High
18	CO_2 losses	PBRs _ Ponds	Depends on pH, alkalinity, etc.
19	Cultivation of algae	Limited to few strains	Versatile
20	Biomass productivity	Low	High

Source: [3,43,79, 81]

4.4. Hybrid production systems

This technique combines distinct growth stages in photobioreactors and as well as in open ponds. The first stage is in a photobioreactor where controllable conditions minimize microbial contamination and favour monocell culture system [89, 90]. Further, the production stage is carried out in raceway pond. In this stage, microalgal cells are exposed to various nutrient stress, which enhances synthesis of the desired lipid product [3].

5. Methods of Recovery of Microalgal Biomass

The fiscal recovery of microalgal biomass still remains as a major challenge. It is documented that, harvesting accounts to 20–30% of the total cost due to small size of microalgal cells (2-20 μm in diameter) and high water content of the broth [43]. Various methods such

as flocculation, sedimentation, flotation, filtration, centrifugation and drying have been under practice for harvesting the biomass.

5.1. Flocculation

It is the most cost-effective and reliable method used for harvesting different species of microalgae. It is achieved by addition of chemicals (organic and inorganic), micro-organisms and rarely by auto-flocculation to form larger clumps, which ease the process of separation (Table.8). An ideal flocculent should be non-toxic, inert and economical. For the recovery of most of the unicellular microalgae cultured in open or raceway pond system, flocculation is used as a pre-treatment step to increase the particle size [74, 91].

5.2. Sedimentation

It is widely used separation technique in wastewater treatment processes. Lamella separators and sedimentation tanks are used for gravity sedimentation. Gravity sedimentation results in high microalgal harvesting efficiency only when preceded by flocculation. Factors influencing particle settling velocity of untreated microalgae are gravity force, particle diameter, density of the medium, density of particle and medium viscosity. It is the most appropriate method due to low capital costs even in large scale operations [74]. However, it is suitable for microalgal species with high sedimentation rates. The advantage of this technique is it is inexpensive, process control is easy with only a requirement of a settling tank and is amenable for large scale biomass harvesting [81].

Table 8: Different types of flocculants used for harvesting microalgae

Sl. No	Method	Advantage	Disadvantage
1	Gravity sedimentation	1. Inexpensive 2. Low energy consumption	1. Not suitable for all types of microalgal species 2. Low reliability 3. Low efficiency
2	Flocculation	1. High recovery 2. Reliable 3. Low energy consumption	1. Flocculants may be expensive 2. Not suitable for all types of microalgal species 3. Time consuming
3	Floatation	1. Does not require addition of chemicals 2. Relatively fast	1. Particle size should be less than 500μm
4	Centrifugation	1. High recovery 2. Corrosion resistance 3. Rapid	1. High energy consumption 2. Expensive 3. Cannot be used for species <30 μm
5	Filtration	**1.** Reliable **2.** Able to harvest species of low density	1. Filters may have to be replaced periodically 2. Membrane fouling & clogging 3. Time consuming 4. Expensive
6	Electrolytic method	1. Inexpensive 2. Low risk of contamination 3. High efficiency 4. No addition of chemicals 5. Reduces operation time	1. Cathode fouling 2. Unsuitable for large scale operations
7	Immobilization	1. More stable 2. High efficiency	1. Expensive 2. Unsuitable for large scale operations
8	Drying	1. No addition of chemicals	1. Requirement for large drying surfaces 2. Risk of material loss

5.3. Floatation

It is a process in which the algal cells are attached to the micro-air bubble surface and are carried on to the surface [104, 109]. Unlike flocculation, floatation does not require addition of chemicals [110]. Hanotu et al in 2012 reported that small bubbles take longer time to rise making them more susceptible to aggregate with the microalgae particles compared to large bubbles [111]. To achieve higher efficiency, the particle size should likely be less than 500μm [112]. Chen et al noted that flotation was more beneficial in microalgal removal than sedimentation and furthermore, it is relatively fast compared to sedimentation [32].

5.4. Centrifugation

The use of centrifugation for biomass recovery and dewatering is considered to be rapid, easy, non-disruptive and high efficiency technique [81, 113]. Cell separation is achieved by increasing the gravitation field subjected to the microalgal suspension thereby concentrating the biomass into a cake with >95% cell harvest efficiency at 13000/g [88]. However, this technique requires high energy consumption and therefore it is not suitable for large scale and commercial scale operations [92, 45].

5.5. Filtration

Filtration is influenced by the size of microalgal cells and the nature of the filter used. Various types of filters are used for harvesting microalgae. Conventional filtration methods such as rotary drum pre-coat filters and press filters are unsuitable for harvesting all microalgal species, as the size range of microalgae range between 2-30μm [92]. Therefore, micro-filtration (pore size ranges from 0.1-10 μm) is appropriate for biomass recovery process. Macro-filtration (pore size is >10 μm) is suitable for flocculated and larger microalgal cell biomass recovery [104]. However, these methods are unsuitable for large-scale operations [114].

5.6. Electrolytic Method

It is another potential approach to separate microalgal cells without the addition of chemicals. In this method, an electric field drives algae to move out of the solution. Water on electrolysis generates hydrogen, binds to the microalgal cells, forms complexes and carries to the surface. Advantages of electrochemical method are highly efficient, versatility and safe. Limitations are high energy consumption and unsuitable for large scale purpose [74, 115].

5.7. Immobilization

Several microorganisms have a natural tendency to attach to surfaces and grow on them [116]. This property is used for immobilizing microbial cells on immobilizing agents such as sodium alginate. Immobilization of the microalgal cultures provides a ready-to-retrieve ancillary platform for biomass recovery [117]. Immobilized biomass can be used for biofuel conversion by thermal or fermentative means. For example, immobilization of hydrocarbon rich microalgae, *Botryococcus braunii, Botryococcus protuberance* on alginate beads yielded a significant increase in chlorophyll, carotenoids, dry biomass weight and lipids during the stationary and resting growth phases compared to free living cells. In addition, the immobilized cells are more stable than free cells.

5.8. Drying

Harvested biomass must be processed immediately after harvest. Dehydration or drying is commonly used to extend the viability depending on the final product required. Various methods of dehydration are sun drying [118], low-pressure shelf drying [118], spray drying [119], freeze drying [120].

Sun drying is the most economical drying method. However, the main disadvantages is time consuming , requirement for large drying surfaces and the risk of material loss [118]. Spray drying is commonly used for extraction of high value products, but it is relatively expensive and can causes significant deterioration of certain algal pigments [119]. Freeze drying is equally expensive, especially for large scale operations, but it is unsuitable for the extraction of oils. Intracellular elements such as oils are difficult to extract from wet biomass with solvents without cell disruption, but are extracted more easily from freeze dried biomass [92, 120].

Table 9: Advantages and disadvantages of different microalgal harvesting methods

Sl. No	Method	Advantage	Disadvantage
1	Gravity sedimentation	1. Inexpensive 2. Low energy consumption	1. Not suitable for all types of microalgal species 2. Low reliability 3. Low efficiency
2	Flocculation	1. High recovery 2. Reliable 3. Low energy consumption	1. Flocculants may be expensive 2. Not suitable for all types of microalgal species 3. Time consuming
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6. Extraction Techniques

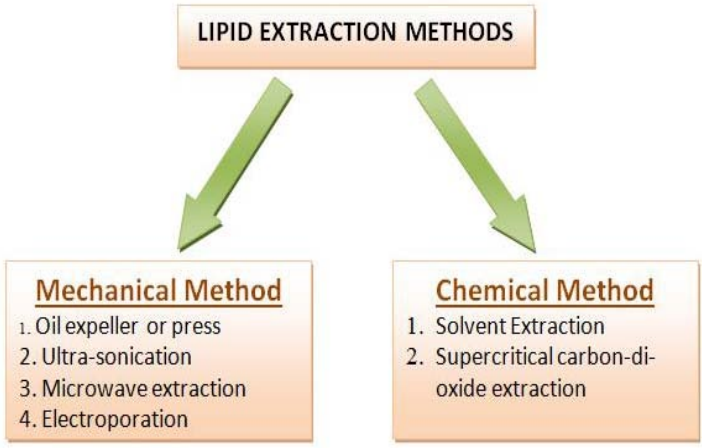

Figure 8: Types of lipid extraction methods

6.1. Press/ Oil expeller method

It is one of the simple, mechanical crushing method commonly used for extracting oil from plant seeds. Oflate, this method is also employed to extract lipid from algal biomass [61]. In this method, high mechanical pressure is applied for crushing and breaking the cells. This results in release of oil contents from the algal biomass. However, high mechanical pressure results in decreased lipid recovery, increased heat generation and choking problems .Oil recovery ranges between 70–75% [121]. To increase the extraction efficiency, occasionally solvents are used. The major drawback is unlike plant seed oil, extraction of oil from microalgal cells is hindered by the rigid cell wall. Furthermore, along with the oil, algal pigments also get extracted. Before conversion to oil, the pigments have to be separated, thus making the entire process cumbersome and expensive.

6.2. Solvent Extraction

Solvent extraction is simple, rapid and inexpensive method compared. The choice of solvent for lipid extraction depends on the type of the microalgae grown. Solvents used should be inexpensive, volatile, non-toxic and non-polar and poor extractors of other cellular components. The most commonly used solvents for microalgal lipid extraction are n-hexane, benzene, diethyl ether and chloroform [122, 123, 124]. Some of the common methods used for the extraction of lipids are Bligh and Dyer method, Soxhlet extraction and Folch et al method [125, 126, 127] .

6.3. Ultrasonication

It is simple, rapid, imparting higher purity to the final product, economical, less energy consumption and can be operated under lower temperature [128]. Ultrasonic waves are produced that propagate in the liquid medium resulting in alternating high pressure and low pressure cycles. During high pressure cycle, the vacuum air bubble produced during the low

pressure cycle ruptures and emits shock waves. This process is known as cavitation [129]. The shockwaves thus produced damage the microalgal cell wall and thereby favours the leakage of intracellular components. In addition, ultrasonic waves aid in the penetration of solvents such as hexane and facilitate the high efficiency transfer of lipids from the cell into the solvents. The disadvantage of this method is cost effective for large scale application [130].

6.4. Supercritical Carbon Dioxide Extraction

It is a promising technology for lipid extraction and could potentially replace the use of traditional organic solvents [131]. In this technique, carbon-di-oxide is compressed beyond its supercritical point (31°C, 74 bar). Now, the supercritical carbon-di-oxide is brought in contact with algal biomass in an extraction vessel. Due to its high penetrating power, it efficiently extracts oil from algae with less solvent residues compared to other extraction methods [132]. Advantages of SCCE extraction are high penetrating power, high efficiency, low toxicity of the supercritical fluid and minimum solvent residues. Carbon-di-oxide generated during the process can be used for the cultivation of microalgae. This gives further value to the process [61]. Disadvantages are requirement of elevated pressure, high capital and operating costs for a high-pressure SCCE [133].

6.5. Microwave Assisted Extraction

Application of microwave assisted lipid extraction in seeds was first established in the mid-1980s. Microwaves are electromagnetic radiation of frequency ranging from 0.3 to 300 GHz. The contact between a dielectric or polar material such as water (present in the microalgal cells) and a rapidly oscillating electric field, produced by microwaves generates heat, thus producing water vapour within the cell. Eventually, it results in cell disruption. It further leads to electroporation effect which promotes cell membrane damage, thus releasing the cellular constituents [134]. This method is relatively safe, rapid and high efficient in extracting microalgal oils under small scale production [103].

Table 9: Advantages and disadvantages of different extraction methods

Sl .No	Method	Advantages	Disadvantages
1	Oil expeller	1. Easy to use	1. Large amount of biomass is required 2. Time consuming 3. Less efficiency
2	Ultra sonication	1. Reduced extraction time 2. Economical 3. Reduced solvent usage 4. Higher penetration power	High energy consumption
3	Supercritical carbon-di-oxide	1. Easy to use 2. Rapid method	3. Cost effective 4.
4	Microwave	5. Economical 6. Safe and rapid method 7. Reduced solvent usage 8. Improved extraction yield	Filtration/centrifugation is required to remove the solid residue
5	Solvent	High efficiency	1. Cost effective 2. Solvent recovery is energy intensive 3. Not rapid 4. Toxic and highly flammable

7. Conversion of Lipid to Biodiesel

7.1 Hydrothermal Liquefaction

It is employed using subcritical water close to its critical point. Under this condition, hydrogen bonding within the water phase is reduced, transforming it from a polar, hydrogen-bonded solvent to a non-polar solvent, capable of extracting and dissolving organic components from the biomass [135]. However, as shown in the phase diagram of water (Fig.9), HTL also requires high reaction pressures to maintain water in the liquid phase and minimise steam formation, in order to prevent the latent heat losses associated with vaporisation [136].

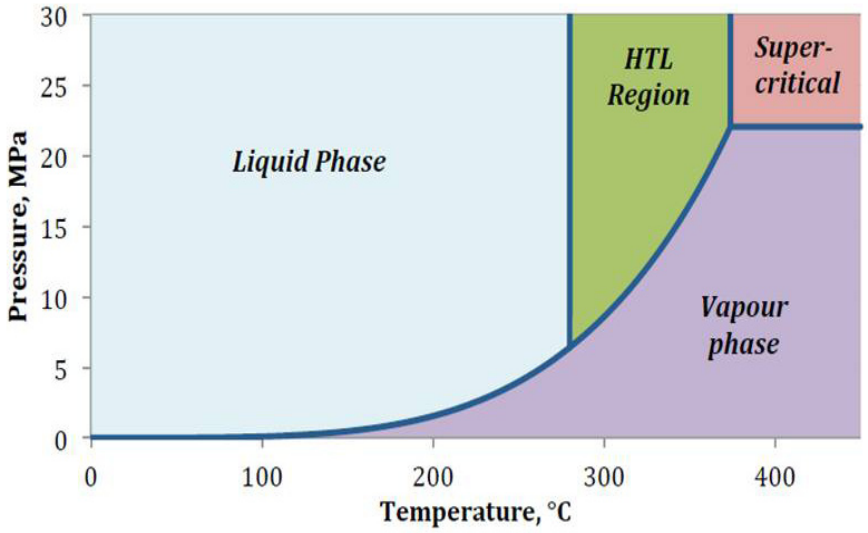

Figure 9: Hydrothermal Liquefaction

Source: [136]

Hydro-thermal liquefaction encompasses four different product phases: solid ash, bio-crude oil, water-soluble compounds and reaction gases. These reactions can be divided into three different stages namely:

First stage: Hydrolysis of the biomass macromolecules (lipids, proteins and carbohydrates) into smaller, water-soluble fragments

Second stage: Rearrangement of the fragments through decarboxylation, deamination and dehydration reactions

Third stage: Dehydration, condensation, cyclisation and polymerization reactions to form the desired bio-oil [135, 137].

The overall process is influenced by temperature, reaction time, biomass concentration and lipid content. The main advantage of this technology is it does not require pre-drying of the biomass and ensures a relatively high product yield [138].

Thermochemical liquefaction of microalgae species such as *Botryococcus braunii*, *Dunaniella tertiolecta* and *Spirullina platensis* yielded 30-80% dry weight basis of oil. This shows that the thermal conversion of biomass to biofuel is an attractive method for liquid fuel production. However, the major disadvantages are reactors for thermochemical liquefaction and fuel-feed systems are complex and expensive [139].

7.2. Pyrolysis

Pyrolysis involves chemically reducing triglyceride to fatty acid alkyl esters (FAAEs) by the application of heat and in the absence of oxygen [19]. In 1986, pyrolysis of microalgal biomass to produce biofuel was first demonstrated in Germany [140].

There are two types of pyrolysis namely slow pyrolysis and fast pyrolysis. In slow pyrolysis, the biomass is associated with liquid fuels, at low temperature (675-775K) and in the presence of air [141]. However, in fast pyrolysis, biofuel is produced in the absence of air at atmospheric pressure, with a relat ively low temperature (450–550°C). In slow pyrolysis, the yield is 15–20% and the main products are char and char-oils whereas, the products of fast pyrolysis are oils and gases with a yield of approximately 70% respectively [142]. Fast pyrolysis has proved to be a promising way to produce bio-oils compared to slow pyrolysis for the following reasons:

(1) Low yield

(2) The viscous bio-oils obtained from slow pyrolysis is not suitable for liquid fuels

(3) Fast pyrolysis process is rapid and less energy intensive

However, the major disadvantage of this process is high equipment cost for separation of various fractions. Also the product obtained was found to be similar to gasoline containing sulphur which makes it less eco-friendly [65].

7.3. Transesterification

It is a multi-step process, wherein, triacylglycerides present in the lipid reacts with methanol in the presence of a catalyst to produce diglycerides, monoglycerides and finally yielding corresponding fatty acid methyl ester (FAME) and glycerol as a by-product (fig.9). Short chain alcohols such as ethanol, propanol, butanol, and amyl alcohol are also used for transesterification. However, ethanol is most frequently used solvent because it is inexpensive and physical and chemical advantages. The production of biodiesel through transesterification can also be achieved by using an alkali catalyst such as sodium hydroxide, potassium hydroxide, sodium ethoxide and an acid catalyst such as sulfuric, sulfonic acid, hydrochloric acid and enzyme catalyst such as lipases (Table.10). Transesterification process is influenced by lipid content, temperature, moisture content, amount of free fatty acids, alcohol etc [115].

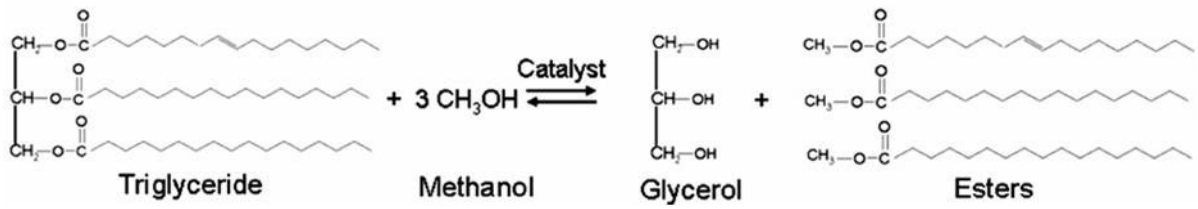

Figure 10: Transesterification reaction of triacylglycerides extracted from microalgal oils for fatty acid methyl ester (biodiesel) production

Table 10: Types of transesterification methods

Sl. No	Types of transesterification	Advantages	Disadvantages	Reference
1	Chemical catalysis	a. Reaction condition can be well controlled b. Large-scale production c. Methanol produced can be reused	a. High temperature b. Energy intensive	[143, 144]
2	Enzymatic catalysis	a. Moderate reaction condition b. High yield c. Eco-friendly d. Small amount of chemicals is required for the process	a. Conversion process is high b. Chemicals hinders the enzymatic activity	[145, 146, 147, 44]
3	Supercritical fluid technique	a. Reaction condition can be well controlled b. Eco-friendly c. Rapid	a. Energy intensive b. Expensive	[148, 149, 44]
4	In situ transesterification	a. High yield b. Rapid c. Eco-friendly d. Economical	a. Energy intensive	[150, 151, 152]

8. Genetic Engineering of Microalgae

Enhanced lipid synthesis and accumulation is pivotal to achieve economic viability of biodiesel production from microalgae. However, such a robust strain remains elusive for researchers even after decades of screening natural strains [153]. Most of the strains known to-date possesses either one or few of the required characteristics. The first pioneer work on genetic manipulation of microalgae was isolation and overexpression of Acetyl CoA Carboxylase (ACCase) from *Cyclotella cryptica*. This enzyme catalyzes a key metabolic step in the synthesis of fatty acid in algae. Although the full-length ACCase gene was overexpressed in yeast and *C cryptica*, no increased lipid production was observed [37]. Many attempts to up-regulate the ACCase encoding gene and other genes in the pathway of fatty acid synthesis failed to achieve anticipated results, showing that direct manipulation of the fatty acid synthesis pathway is not a hopeful strategy. However, up-regulation of TAG assembly genes, such as glycerol-3-phosphate acyltransferase or diacylglycerol acyltransferase had enhanced oil content in many plant seeds suggesting that enzymes in TAG assembly pathway are interesting candidates for genetic manipulation to enhance lipid biosynthesis in microalgae [47].

Table 11: Various studies on genetic engineering of microalgae for lipid synthesis

Sl. No	Target protein	Host	Type of medication	Gene source	Primary phenotype change	Reference
1	Acetyl-CoA carboxylase	*Cyclotella cryptica*	Nuclear over expression	Endogenous *Navicula saprophila*	No increase in total lipid accumulation	[154]
2	Malic enzyme (ME)	Chlorella pyrenoidosa	Overexpression of the gene PtME	Phaeodactylum tricornutum, a diatom	Lipid content increased by 3.2 fold	[155]
3	Malic enzyme (ME)	*Phaeodactylum tricornutum*	Putative malic enzyme gene	Endogenous	Lipid content increased by 2.5-folds	[156]
4	Pyruvate dehydroganase kinase	*Phaeodactyllum tricornutum*	Antisense Cdna	Endogenous	82% increase in neutral lipids	[157]
5	Malic enzyme	*Phaeodactyllum tricornutum*	Nuclear overexpression	Endogenous	2.5-fold increase in total lipids	[156]
6	Lipogenesis transcription factor	*Chlorella ellipsoidea*	Nuclear overexpression	Soybean	52% increase in total lipids	[158]
7	Overexpression of DGAT enzyme	*Chlamydomonas reinhardtii*	RNAi	Endogenous	34% rise in TAG production	[159]

9. Commercialization of Microalgae

Oflate, many attempts have been done to commercialize microalgal biofuels. In 2010, the U.S. Department of Energy (DOE) announced an investment of up to $24 million for three research groups aimed at commercializing biofuels derived from algae. The Sustainable Algal Biofuels Consortium of Mesa, Arizona, led by Arizona State University was funded with $6 million to investigate biochemical conversion of algae to biofuels and other value-added products. Another team led by the University of California, San Diego, is received $9 million to develop algae as a robust biofuel machinery. Several companies are also attempting to commercialize microalgal biodiesel. For example, in July 2009, Exxon Mobil Corporation announced an alliance with Synthetic Genomics Inc. to develop next generation biofuels from photosynthetic algae. In U.K., Carbon Trust Company has invested millions of dollars in the commercialization and utilization of algae-based biofuel through Algae Biofuels Challenge project. The U.K. government announced it would contribute to the further funding of this project. Although the investments in biofuel production from algae are being increased worldwide, several challenges must be tackled before commercial-scale production of biofuels from algae can be achieved [79, 161, 162].

10. Conclusion

Microalgae are considered as the most promising microbial cell factories for biodiesel production. It is the only renewable biodiesel that can potentially replace liquid fuels derived from petroleum Adequate oleaginous microalgal strains with increase tolerance to varying environmental stress can be grown in photobioreactors or open ponds on large scale for biodiesel production. However, new technologies have to be developed and improved, involving the harvesting of microalgal biomass, dewatering, extraction of microalgal oil, transesterification and downstream processing. The main hurdle of microalgal biodiesel production is lowering the cost to make it competitive with petroleum derived fuels. Producing low-cost microalgal biodiesel requires primarily improvements to algal biology through genetic and metabolic engineering. However, these technologies are still in the infancy stages and most have not been applied on a commercial scale. Therefore, further research in the development of novel upstream and downstream technologies will benefit the commercial production of biodiesel from microalgae.

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Advances in Biotechnology

Chapter 4

Biofilm Formation and its Role in Antibiotic Resistance

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1. Introduction

Most of the life forms in the world can develop skills for their continued existence against a constantly changing and challenging environment. Amongst all the organisms, bacteria show a tremendous adaptation, by natural selection through transformation crafting genetic variants [1] and show survival instincts in many ways. They can form surface attachments, three dimensional edifices that are sustained by self-synthesised extracellular polymeric matrix. This consortium of cell-cell interaction can be described as biofilms [2], which represents the defence and communication system of a bacterial community. Naturally, biofilms are constructed by a diverse group of microorganisms like *Pseudomonas aeruginosa*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Streptococcus mutans* which co-exists as a community challenging the hostile environment created by the host defense mechanism followed by the resulting antibiotic exploitation in order to eradicate the formed biofilm [3]. The transmission of a microbial invasion to a chronic pathological condition in not less than 65%, percentage is associated with biofilm formation especially in lung infection in cystic fibrosis, peridontitis of the teeth, middle ear infections, osteomyelitis, wound infections and nosocomial infections in prosthetics of joints, intravenous catheters, urinary catheters and stents [4,5].

Microbes restrain from its planktonic form to sessile mode and pin down to a location to grow into a microcolony like assembly concealed in a polymeric matrix organically synthesised. This dynamic environment evolves the siocio-microbial association a characteristic

physiological and behavioural modification conferring antibiotic resistance as a survival strategy. This alarms the WHO which recognised the antibiotic resistance is a serious problem not only for the human population but for the other organisms the domestic and wildlife. Indeed, it is difficult to restrain antibiotic resistance to one ecological niche but tends to spread universally through horizontal gene transfer [6,7]. Antimicrobial agents are the only existing therapy for treating microbial infections, infections; nevertheless, they could not completely eradicate biofilms conferring persistent infections in living organisms. The biofilm architecture comprising high cell densities protected in an exopolysaccharride matrix requires higher concentration of antibiotics approximately 10-1000 times than that of their planktonic counterparts. Administration of antibiotics in such heavy doses is in itself impossible due to the complications associated with the cellular damages in course of the metabolism and elimination process [8].

2. Stages of Biofilm Formation

The formation of biofilm is a gradual process and independent of the phenotype of the host microorganism [9]. Adhesion, growth, motility, and extracellular matrix production are the steps involved in the development of biofilm which is divided into several stages that are cyclic in nature. Stage 1 is a phase of reversible adhesion of the microbial cell to a surface which is mainly driven by motion, gravitational forces and hydrodynamic forces [10]. It has been studied recently that rough and hydrophobic surfaces such as bone, cartilage and heart valves as well as foreign body implants like catheters and Orthopaedic devices are mostly preferred for surface adhesion. They are highly influenced by pH, temperature, nutrients and their concentration, oxygen concentration osmolality and iron levels [11]. Stage 2 involves production of signals for communication between cells which helps in their growth. Stage 3 is a primary maturation phase where the production of an extracellular polysaccharide matrix is enhanced and motility is gradually decreased. Stage 4 is a phase of cell dispersion in which some bacteria leave the biofilm due to planktonic phenotype development. This results in release of free floating cells capable of reforming biofilm in a different place [12].The consortium of microorganisms within a hydrated environment possibly Exhibits a survival strategy against predation **(Figure 1)**., defence (protection from toxins in the host), colonisation (sequestration in a nutrient rich media), community (utilization of public benefits in a multispecies environment), d efault mode of growth (bacteria normally grow as biofilms only).

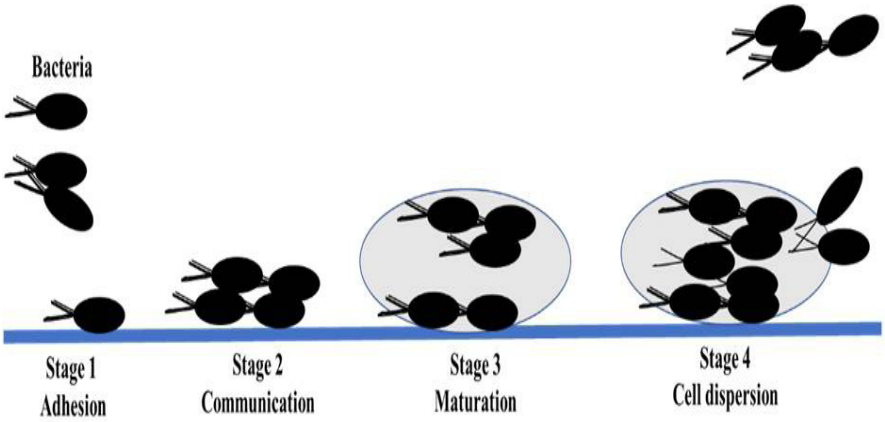

Figure 1: Stages of Biofilm formation

3. Role of EPS in Biofilm Formation

The polysaccharide component, also known as exopolysaccharide (EPS), provides the biofilm with benefits including attachment or adhesion to biotic or abiotic factors, architecture and protection from environment especially from dehydration [13,14,15]. The environmental stress on the biofilm, the maturation period of biofilm and the type of microorganisms are responsible for the constituents and mass of EPS [16]. EPS contributes 50-90% of the entire organic matter found in the biofilm [10]. The attachment of biofilm to the *invitro* and *invivo* substrate like prosthetics and endothelial valves of tissues respectively? is enhanced through the divalent cations present in the outer membrane of a bacterium; the divalent cations like Ca2+, Na+ and Mg2+ aid in maintaining the stability of the structures in the outer membrane [17]. In gram negative bacteria the polysaccharides that constitute the EPS are either of neutral or of negative charge which associates with the divalent ions strengthening the biofilm organisation while the gram positive bacteria, has a positive charge and hence doesn't involve ions presenting a compositional variance of the EPS [10]. The surface to which the biofilm attaches itself and the degree of adhesion of biofilm are directly related to each other; an uneven surface which is hydrophobic in nature is advantageous since the unevenness allows the biofilm to be protected by providing confined spaces [10].

3.1. Chemical Composition of EPS

EPS is made up of a variety of constituents ranging from carbohydrates [18], proteins [19] nucleic acids [20], humic substances [21], organic bases (hydroxyl groups) and organic acids (carboxylic groups) [22]. Presence of polysaccharides, proteins and nucleic acids in the EPS was well evaluated by NMR and FTIR analysis [23] Pal and Paul (2008) confirmed the presence of carbohydrates, proteins, nucleic acids and small amounts of uronic acid in EPS collected from a waste water treatment plant [24]. Sand and Gehrke [25] reported the presence of neutral sugars and lipids in *Acidithiobacillus ferrooxidans*. The EPS constituents like polysaccharides (dextran and kefiran) from lactic acid bacteria, *Weissela, Fructobacillus, Lactococcus* and *Streptococcus* are commercially promoted [26]. Guo-Ping Sheng [27]

concluded that extraction methods are vital in determining the amount of EPS. The total biofilm enzyme activity elucidation could be overlooked because of the disrupted matrix suspensions of the older biofilms of more than 30 days old and intact biofilms of young cells. Hence forth appropriate extraction methods are needed in the assessment of biofilm studies. The composition of EPS depends on the expression of the genes, environment and also the available or attached substrate [28,29]. *Staphylococcus epidermidis* was reported to produce polysaccharides responsible for binding to the medical devices, where the similar kind of polysaccharide i.e. poly-N-acetylglucosamine is produced by *Staphylococcus aureus* [30,31]. There are reports for production of exopolysaccharide like β-1-6 linked 2-amino-2-deoxy-d-glucopyranosyl residues by *S. aureus* [31,32]. Bacterial colonisation studies could also reveal the enzyme activity which deciphers thein metabolically active state of cells. The secretion of enzymes and molecules into the polymer matrix reveals the 'altruistic' behaviour where as the liberated molecules are not only used by the producer but also by every member of the microcolony. Role of these molecules in the biofilm could leave us a clue in spotting a better biofilm target [(32)].

3.2. Applications of EPS

The EPS secreted by microorganisms is employed in various fields such as food, industrial, mining & metallurgy [33] pharmaceutical, biomedical and the diverse structure of EPS has allowed it to be useful in the fields of bioremediation and bioleaching [26] rather than the preceding physical and chemical methods. EPS is responsible for the removal of toxic components from the environment by flocculation [24] or by metal chelation [21] and EPS showed an effect on termination of sulphates [25] as well as organic matter dissolved within aquatic systems [34]. Bioremediation through biofilms is more efficient than planktonic bacteria as biofilms are capable of adapting to the critical environmental conditions [10]. EPS has also been reported to remove remazol (dye) from effluent efficiently, due to its tremendous biosorption ability [35].

4. Role of Biofilm in Antibiotic Resistance in Bacteria

Antibiotic resistance is a phenomenon where Pathogenic bacteria cannot be inhibited by any one or more antibiotics. In such cases the bacteria become resistant to the antibiotics and continue to persist even in the presence of antibiotics. The resistance may be due to biochemical or evolutionary routes that confer resistance to the antibiotic used [6]. The evolutionary factors may influence antibiotic resistance through the formation of a biofilm. Bacteria within a biofilm correspond to a fundamental survival mechanism in which the organisms are protected through various biochemical pathways [37]. Multi drug resistant organisms have a major impact on public health as they exhibit resistance against a wide range of antibacterial agents [38]. Biofilms are responsible for almost 60% of nosocomial diseases related to contact lenses,

pacemakers, prosthetic joints, mechanical heart valves, central venous catheters, urinary catheters, prosthetic devices and orthopaedic devices [39]. These devices act as substrates for biofilm that causes infections and thus demands regular removal and replacement of these devices [40]. Cells from a disrupted biofilm become susceptible to antibiotics when grown in a planktonic state [41,42].

4.1. Slow Permeability of Antibiotics

It is regarded that exopolysaccharide secretion prevents the inlet of antibiotics into cells [42]. Various strains of *Pseudomonas aeruginosa* produce alginate, a negatively charged polysaccharide which helps in maintaining the integrity of the biofilm and further more prevent the entry of positively charged antibiotics such as amikacin and gentamicin [43]. *Staphylococcus aureus* involves in formation of PIA (polysaccharide intercellular adhesion) which helps in the gathering of nutrients during biofilm formation and plays a significant role in the development of biofilm related infections therefore escalating its resistance to antibiotics [44]. The cell membrane of *Staphylococcus epidermis* is surrounded by a glycoprotein polysaccharide called glycocalyx which effectively reduces the susceptibility to various antibiotics [45,46]. The slime secreted by *S. aureus* and *S. epididermis* decreases the susceptibility of the organism towards the activity of glycopeptides and pefloxacin [30,46,47]. De Beer et al [48] confirmed that vancomycin sufficiently penetrated *Staphylococcus epidermidis* biofilm but eradication of biofilm was not favoured. *Invitro* studies are also reporting that biofilms surrounded with polysaccharides possess additional resistance towards any harsh environment [49]. Even the host mechanism does not impact the defence gained by the biofilm towards antibiotics.

4.2. Alteration of Antibiotics

4.2.1. Alteration of efflux pumps

Alteration in pumps lead to infiltration of various antibiotics into the biofilm, which is caused by mutation of genes or enzyme mediated drug modification [37,50]. Singh et al [51] speculated that bacteria enter a phenotypic differentiation that confers resistance either by modification of drug binding sites or through expression of efflux pumps. Bacteria can also obtain supplementary resistance from different organisms through mobile genetic elements [52]. Mutation in genes coding for porins leads to resistance against β-lactam antibiotics [53,54,55]. Mutation in five major classes of efflux pumps leads to drug resistance and they are: ATP Binding Cassette (ABC) superfamily, the Major Facilitator Superfamily (MFS), the Multidrug and Toxic-compound Extrusion (MATE) family, the Small Multidrug Resistance (SMR) family and the Resistance Nodulation Division (RND) family [56]. Multidrug efflux pump expels chemical agents and also the antibiotics from the cells. Up regulation of mar operon in *E. coli* is associated with the multidrug efflux pump AcrAB [50]. MexAB–OprM and MexCD-OprJ pumps found in *Pseudomonas aeruginosa* confer fluoroquinolone resistance [43,50] and

it also expels few antibiotics such as β-lactams macrolides, trimethoprim, chloramphenicol, novobiocin and tetracycline [57]. Few efflux pumps belonging to the resistance nodulation division family such as AcrAB–TolC, MexAB–OprM, CmeABC and MtrCDE enhance cohesion and colonisation of biofilms on the host surface [56].

4.2.2. Alteration of Antibiotic Binding Site

Alteration of the binding site or the target sites where antibiotics bind is commonly exhibited by bacteria. Mutation at enzymes like RNA polymerase and DNA gyrase leads to resistance against enzyme inhibiting antibiotics [58]. Mutation is the major cause for this alteration. One example is the Mutation in the rifampin binding site i.e. RNA polymerase which leads to resistance against rifampin [59], which is observed in *Mycobaterium tuberculosis* [60].

4.2.3. Inactivation of Antibiotics

Enzymes produced by the microorganisms are responsible for the inactivation of antibiotics. From past century there are more examples, even penicillin is cleaved by β-lactamase enzymes. As the microorganisms have evolved there are more mechanism which conferantibiotic resistance including integrons (gene expression cassettes) [61]. These enzymes convert the antibiotics by either doing one or more modification as follows – a) adenylation b) phosphorylation and c) acetylation. Multiple aminoglycoside modifying enzymes are reported to possess transferase activity against aminoglycoside and leads to resistance against aminoglycoside [62].

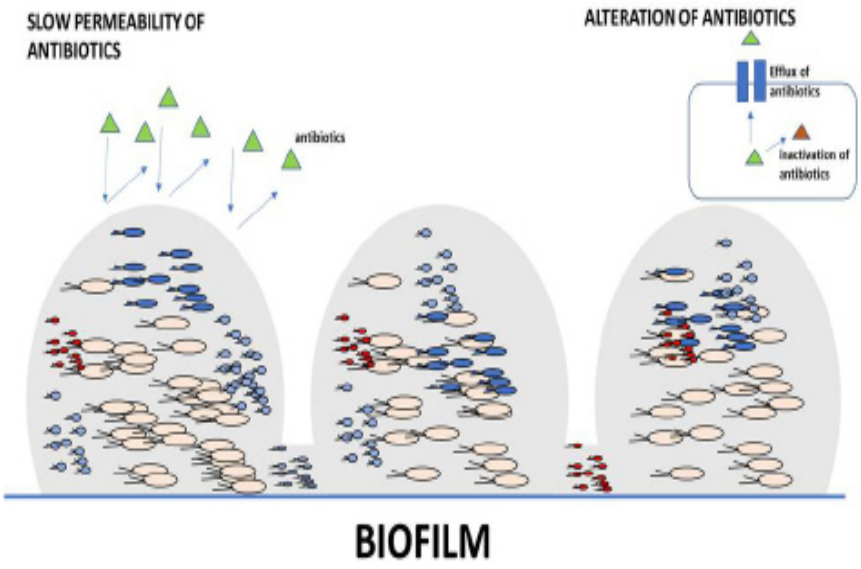

Figure 2: Antibiotic resistance exhibited by biofilm

5. Quorum Sensing and Biofilm Formation

The regulation of cell relying on its mass is termed as “Quorum Sensing” [63], the way bacteria communicate among themselves. This signalling is believed to be responsible for

growth, virulence, biofilm formation [2], sporulation [64], pigment production [52], antibiotic resistance and symbiosis and increases the pathogenicity of the microorganism [65]. Gram negative bacteria utilise N-acyl homoserine lactones for the signalling, which is produced by acyl carrier protein (ACP) [66,67], where Gram positive bacteria use peptides for quorum sensing [68]. *Pseudomonas aeruginosa* uses rhl genes for signalling [69] because lasI gene is responsible for production of N-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL), and rhl is responsible for the production of *N*-butyryl-L-homoserine lactone (C4-HSL).In *Pseudomonas aeruginosa* approximately 4% of the genes out of 6000 genes function by the mechanism of quorum sensing [70]. The expression of the exoproducts in *P. aeruginosa* like elastase Las A, elastase Las B, exotoxin A and alkaline protease was initially regulated by Las RI system [70]. In *P. aeruginosa* quorum sensing is also controlled by the LuxRI homologues and VsmRI. The synthesis of N-Butanoyl-Lhomoserine lactone (BHL) is directed by RhlI [69]. The expression of rhlAB, an operon encoding rhamnosyltransferase essential for the production of rhamnolipid is due to the interaction of acyl HSL with RhlR. Rhamnolipids are bio surfactants which help in reducing the surface tension [71]. Sigma S encoding RpoS protein helps in the expression of many activities that are known to be regulated by the Las and Rhl regulons [72].

Staphylococcus aureus causes nosocomial infections worldwide. Biofilm formation in *Staphylococcus aureus* allows the attachment of cells to a biotic or abiotic surface with the help of adhesions. Multiplication of the cells in the adhesive matrix gives rise to many layers which are associated with the production of extracellular factors, as well as the polysaccharide intercellular adhesion component [73]. The Quorum sensing system of *S.aureus* is different from that of *Pseudomonas aeruginosa* acyl homoserine lactone system. The accessory gene regulator (agr) locus is responsible for the quorum sensing system in *S.aureus* [74]. The virulence contributed by the agr system varies with the type of infection model used [75]. The virulence associated with agr is due to four proteins AgrB, AgrA, AgrC, and AgrD which are encoded by RNAII [76]. Agr can up-regulate 104 genes and down-regulate 34 genes that are involved in quorum sensing [77]. After exponential phase the agr locus directs the expression of RNAII and RNAIII transcripts through two promoters P2 and P3 [76]. At stationary phase, the agr prevents the expression of cell surface proteins and activates expression of the genes involved in the secretion of exotoxins and tissue degrading mechanism [78]. The agr locus seemingly affects several extracellular and cell wall associated protein when a transpose on (Tn551) is inserted [79]. An octapeptide is generated by AgrD and AgrB which at extracellular threshold concentration activates AgrC and AgrA responsible for the regulation of a two- component regulatory pathway [76,78].

In *Escherichia coli*, two major components of cpx signalling system are Cpx A and Cpx R. Among these, CpxA is a sensor kinase, phosphatase, involves in bacterial conjugation and

also stabilises cell surface interactions [80]. NIpE, an outer membrane lipoprotein initiates Cpx signalling system after interaction with surface and upregulates pili mediated surface adherence mostly to hydrophobic environment and regulates OmpF and OmpC [80,81]. Increased osmolarity activates EnvZ/OmpR signalling system which further produces phosphorylated OmpR and results in better adherence of the cells to the surface [82]. Phosphorylated OmpR indirectly regulates csAb operon and it codes for the structural subunits of curli, which is specialised form of pili [83]. Phosphorylated OmpR also positively regulates transcription of adrA gene which is involved in production of cellulose, which is a part of EPS in *E.coli* and *Salmonella typhirium* [84]. The EnvZ/OmpR signalling system has been found to be conserved among various bacterial species [85]. It has been observed that the EnvZ/OmpR signalling system induces surface adherence only in response to moderate increase in osmolarity while drastic rise in osmolarity impedes biofilm formation in a few species like *E. coli, Pseudomonas fluorescens* and *Streptococcus gordonii* [82]. Vibrio fischeri is a gram negative bioluminescent marine bacterium which is considered to be the finest model to understand the process of Quorum sensing. Bioluminescence is a cell population density based mechanism. The multifactorial mechanisms which are responsible for bioluminescence is well understood [86]. In *Vibrio fischeri*, the genes responsible for bioluminescence contain two chromosomes out of which the luxCDABEG gene present on the second chromosome is an integral part of the operon which is responsible for all the structural components necessary for bioluminescence [87]. The enzyme luciferase encoded by luxA and luxB is responsible for bioluminescence; it coordinates simultaneous oxidation of a long chain aldehyde and reduction of flavin mononucleotide. The fatty acids required for luminescence is derived by the diversion of fatty acyl groups from the fatty acid biosynthesis pathway by luxD [88]. LuxI and LuxR control the luciferase operon. In order to initiate luminescence, Acyl-homoserine lactones (AHLs) produced by LuxI and AHL coinducers produced by LuxR (DNA binding transcriptional activator) is required [89].The produced AHL molecules constantly diffuse in and out of the cell membrane increasing the concentration of cell population, once the threshold concentration is reached the AHL bound to LuxR activates thereby transcribing the luciferase operon which results in the emission of light [90,91].

6. Conclusion

Over the years bacteria have evolved beyond our imagination. The impact of bacterial evolution on humans is vivid from the increasing number of untreatable diseases. Bacterial communication systems have advanced creating a new era for bacteria. But we have grasped the evolution pattern and the signalling involved in communication systems. Present day advances in various fields of science and medicine has extended our knowledge on quorum sensing systems and technology has given us limitless opportunities to explore. Therefore, our aim is to develop alternatives to antibiotics (supplements which act on biofilm formation) or

discovering new antibiotics will help us to overcome the impact of Quorum sensing.

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Advances in Biotechnology

Chapter 5

Recent Advances in Cardiovascular Diseases and Treatment

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Abstract

Cardiovascular diseases (CVD) are one among the most common causes of death worldwide. There are plethora's of events leading to cardiovascular pathophysiology. Despite, recent advancements in the treatment of cardiovascular diseases, it remains the number one cause of death in the world. While traditional risk factors partially account for the development of CVD, other novel risk factors have recently been implicated. Specifically chronic inflammation has been postulated to play a role in the development and propagation of this disease. Reactive oxygen species (ROS) generated during excessive oxidative stress are the one among responsible for the various inflammatory events in cardiovascular disorders including atherosclerosis, cardiac hypertrophy, cardiomyopathy heart failure, ventricular remodelling, ischemia/reperfusion injury and myocardial infarction. In the last decade, significant advancements in CVD treatment have been made and achieved some curative effects as well. The existing treatment is medical, surgical or a combination of both depending on the extent if severity and clinical presentation of CVD. The collaboration of different science disciplines likely biotechnology and tissue engineering has led to the development of novel therapeutic strategies: Stem cells therapy, Nanotechnology, Robotic surgery and Drugs. These treatment modalities show promising effects in management of CVD and associated conditions to larger extent.

Keywords*:* Cardiovascular Diseases; Robotics; Nanotechnology; Stem cells

1. Introduction

Cardiovascular diseases are diseases of circulatory system which involves either one or both of the heart and blood vessels (arteries veins and capillaries). The recent advancements in CVD and its physiology have led to a subsequent decrease in the mortality rate in the aged population. [1]. However CVD remains one of the leading causes of death worldwide [2]. There has been a greater focus in research aimed at all aspects of CVD in the last decade. In the recent past there has been significant progress made in developing novel strategies for patients of CVD and its associated complications. These strategies range from new therapeutic targets, drugs to robotic surgery and nanotechnology. This article will summarize the literature evidence on the recent advances ment in cardiovascular disease research with respect to therapeutics and biomarkers. The topics will cover the following headings: robotic surgery, nanotechnology stem cells and other basic research related advancements.

1.1. Robotics

Robotic interventions, the role of non-invasive imaging surgery and radiotherapy have been in use for more than a decade. In cardiology this technique is utilized for surgeries that are dependent on being able to see the exact location within the heart in 3D and having mechanical assistance such as computer-assisted technology or robotic assistance or better imaging and most likely both of these [3]. Further on as this matures and we will have better software technologies, there'll be important improvements in Tran's catheter ways of addressing the cardiac disease. In recent times specifically 2018 onwards this technique have been in use for surgeries like mitral valve repair, coronary artery bypass graft and septal defect closure including transesophageal to assess structural heart disorders especially to guide therapeutic decisions and procedures. The technology is fast evolving with reports emerging about their potential applications in percutaneous coronary interventions and atrial fibrillation ablation [4]. Robotic guided surgery has the potential to limit this radiation exposure. In addition they can also reduce contrast-induced nephrotoxicity and associated mortality in patients [5]. Robotics provides the operator with advantages such as improved ergonomics precision and sometimes shortening of intraoperative time [6]. There have been reports that robot-assisted surgery can shorten the duration of patients hospital stay and will improve patient perception of disease [7].

In terms of patient-related outcomes the robotic-assisted surgery has potential benefits as it can accurately measure the size of the lesions (which can be miscalculated using 2D angiography) which could improve long-term health benfits. Hence they reduce radiation exposure for the surgeon and the patient as well as improve precision by rendering accurate measurements of lesions. In a multicentre study published by Weisz et al. a percutaneous

coronary intervention was performed to patients with coronary artery disease [8]. They used similar success criteria (measured in terms of less than 30% residual stenosis along with the absence of major cardiac complications) and reported a 97.6% rate of success (164 patients) [9]. They also reported a significant reduction (95%) in operator radiation exposure [9]. Although there are reported benefits for robotically assisted bypass grafting, high costs and long learning curves have slowed down its progress towards becoming used routinely.

1.2. Nanotechnology

Nanotechnology has been revolutionizing several fields of medicine. It involves the engineering of nano-scale molecules with distinctly different properties than bulk molecules of the same composition. These inherent differences provide distinct benefits which are strong reasons for the boom in nanotechnology research. This technology has been studied in CVD for its potential benefits in medical [non-invasive and invasive] treatment modalities, drug delivery applications, percutaneous coronary interventions gene therapy and coronary artery bypass graft [10]. Nanotechnology have shown potential benefits when used in percutaneous coronary intervention. They have been studied for their ability to release drugs as well as promote healing and reduce restenosis. Several nanoparticle-based antithrombotic agents have been tested for their potency. D-phenylalanyl-l-prolyl-Larginyl-chloromethyl ketone is a potent antithrombotic agent, that is rapidly cleared from the body thus limiting its clinical use [11]. When combined with a perfluorocarbon-core nanoparticle it has been shown to have improved antithrombotic action as shown by Myerson et al. in an animal model study. Peters et al. on the other hand used hirudin with fibrin binding micellar nanoparticles which exhibited greater targeting of fibrin clots in vivo [12]. Collagen IV nanoparticles have been tried in an animal model study and were shown to improve collagen formation while reducing oxidative stress by mimicking Annexin A1 (glucocorticoid regulatory protein) [13]. Nano modifications have also helped research scientists in targeting specific drug delivery of collagen IV chondroitin sulphate tissue factor or stents and several nano-coatings in the form of hyaluronic acid (which carries plasmid DNA) nano-biohybrid hydrogel (which carries Tat peptide and DNA) and poly lactic-*co*-glycolic acid nanoparticles (which carries PDGF receptor-β antisense RNA) have been extensively studied in animal models with promising results [14]. Nanotechnology has led to an interesting and promising direction in the treatment of CVD. It has shown promising potential in delivering drugs that are otherwise limited by their pharmacokinetics. Its potential application in stent and gene based therapy are useful for future therapeutics based on these modifications. Further randomized controlled trials need to be conducted to establish strong evidence to support the use of these newer technologies for CVD treatments.

1.3. Stem Cells

Stem cells technology have emerged as an important research target on developmental

morphological and physiological processes that govern tissue and organ formation, maintenance regeneration and repair[15]. Human heart is largely incompatible in replenishing or regenerating lost cardiomyocytes [16]. The therapeutic applications of stem cells is a promising and rapidly emerging branch of regenerative medicine in which stem cell-based treatments could be applied to treat and cure many aggressive and lethal diseases in humans [17]. Recent investigations were carried out with ex vivo expanded or differentiated embryonic stem cells and stem cell-derived from fully functional progeny as well as adult stem/progenitor cells. These have provided accumulating evidence supporting their potential role in the treatment of various genetic and degenerative disorders [18]. Research in CVD has shown to replenish myocardial damage by increasing the blood supply during ischemic conditions of the heart. In recent scenario both vascular growth factors and stem cells have generated a lot of interest for treatment in CVD subjects [19]. The apex and atria of the heart constitute the homing sites of cardiac stem/progenitor cells (CSCs), that are able to give rise to three major cell types of the myocardium-cardiomyocytes, smooth muscles and vascular endothelial cells-in physiologic and pathological conditions [20]. The rationale behind this therapeutic approach is to improve the blood supply to ischemic areas of the heart by stem cells and promote cardiac cell regeneration. This can be achieved in one of two ways: by a direct effect of the stem cells or by paracrine factors secreted by these stem cells [21]. In this regard hematopoietic stem cells have been of great interest especially for mononuclear cells and endothelial progenitor cells. Studies conducted using these cells for various forms of ischemic heart disease (such as acute myocardial infarction (MI) and chronic ischemic heart disease) have been contradictory although some studies have demonstrated a beneficial effect in such patients [22]. Adipose derived stem cells are another form of stem cells utilised for studies. A novel alternative is the creation of induced pluripotent stem cells of which adult cells are transformed into pluripotent stem cells similar to embryonic stem cells [23]. Although it offers a promising alternative, concerns of cancerous transformation of the undifferentiated stem cells have to be taken into account, before they can be tried in human subjects. The stem cells studied in cardiovascular research ranged from bone marrow to adipose tissue to skeletal muscle stem cells. Bone marrow (BM) - derived mononuclear cells are the most readily available cells for transplantation in the body. They are easy to identify based on their cell surface markers and can be isolated from the bone marrow [24]. However their therapeutic potential is low since the harvested cells contain a multitude of cells with a small proportion of stem cells [25]. BM stroma and the vascular walls of peripheral tissues also contain the multipotent EPCs and MSCs localized in perivascular niches that are able to generate mature endothelial cells and diverse mesenchymal cell lineages including osteoblasts, chondrocytes, adipocytes and myoblasts [26]. The BM and vascular wall-resident and circulating EPCs, as well as EPCs, derived from ESCs fetal liver and adult stem cells present multiple important clinical interests. EPCs can be utilised to treat diverse vascular disorders because of their significant high migratory potential through blood and their capacity to differentiate into new endothelial cells that can contribute to promoting

neo-angiogenesis and endothelium repair at distant sites of organ or tissue damage.

The adipose derived stem cells can be surgically harvested from adipose tissues. They are more abundant in comparison to the bone marrow-derived cells. This drastically reduces the time and cost involved in laboratory procedures to culture them for clinical use [27]. The pluripotent stem cells have a high potential for transformation. Although embryos represent the most obvious source of stem cells, their use has ethical concerns and is in debate. Additionally these cells could potentially face rejection when transplanted to a recipient. However it is possible to reprogram adult cells and transform them into pluripotent cells (similar properties as embryonic stem cells) thereby being called induced pluripotent stem cells. These cells can be auto-transplanted and therefore can not be rejected. However due to their transformation potential, unless closely regulated they can undergo teratomatous (derived from all three germ layers) changes in the body [28]. Due to the risk of teratomatous changes this area of research requires more work before they can be considered safe for human trials. Another interesting source of stem cells are cardiac stem cells. Cardiac stem cells (CSC's)or their further differentiated progeny which represents a cell replacement therapy of aged or dysfunctional CSCs and regeneration of cardiomyocytes and coronary vessels is emerging as an area of great interest to many researchers. The experimental and clinical studies have shown promising results [29]. However further research is needed to understand the exact mechanisms of action and the ideal source of stem cells to derive optimum benefit and to further add our understanding.

1.4. Drugs

Drugs for CVD patients (such as hypercholesterolemia) has been statins and fibrates though they are capable of bringing curative effects but are lifelong dependent medications. Recent research has led to various drug developments for CVD patients. One such class of drugs referred to patients suffering from CVD are oral antithrombotic medications such as aspirin and clopidogrel [30]. Oral anti-coagulants group consists of the drugs: ximelagatran, darexaban dabigatran, rivaroxaban and apixaban [31]. Of which dabigatran, edoxaban, rivaroxaban apixaban are FDA approved for clinical use. Dabigatran is a competitive inhibitor of thrombin while edoxaban, rivaroxaban and apixaban are inhibitors of clotting factor Xa. However use of dabigatran in CVD patients was confirmed in phase 2 trial for ischemic events in patients at higher doses of the drug (110 and 150 mg) but with increased bleeding risk [32].

An important protein that controls the regulation of LDL (which is a key regulator in hypercholesterolemia) is proprotein convertase subtilisin/kexin type 9 (PCSK9) [33]. They function to reduce the number of LDL receptors thereby decreasing LDL cholesterol levels in the blood [34]. Another major drug which could act as blocker for PCSK9 is Alirocumab which is a monoclonal antibody (produced by recombinant DNA technology) [35]. The studies with

Alirocumab reported a reduction in LDL cholesterol levels ranging from 28% to 65% depending on the route of administration [36]. Since high levels of LDL levels are linked to CVD the use of Alirocumab reduced adverse cardiovascular events by 15–48% [36]. Another class of drugs recently studied for the treatment of heart failure is the angiotensin receptor-neprilysin inhibitor (ARNi) which contains a combination of sacubitril and valsartan commonly referred to as the LCZ696 or ARNi [37]. The valsartan portion is a drug of the angiotensin receptor blocker family as well as angiotensin II receptor antagonist, while the sacubitril component is neprilysin inhibitor [38]. This drug is proven treatment for heart failure than Angiotensin-converting enzyme (ACE) inhibitors [39].

2. Conclusion

Research progress has led to significant advancements in therapeutic approach despite cardiovascular diseases remain one of the most common causes of mortality and morbidity worldwide. Recent significant inter-collaborative efforts of researchers, clinicians and other health professionals have led to multi-faceted and novel strategies to be developed for CVD and its treatment. Though some of these strategic interventions have strong evidence supporting their clinical use while others still in the experimental trial stage. The early evidence are being available for some of these novel treatment modalities and the results are promising and hold the potential to become alternatives to current treatment options in the future. Since we are dwelling in the era of evidence-based medicine and treatment perspective, further evidence in the form of clinical trials and long term follow up studies are much needed before these novel strategies enter into mainstream treatment practice. With sustained continued efforts the future for CVD therapeutics looks substantially promising.

3. Abbreviations

CVD	Cardio vascular disease
ARNi	Angiotensin receptor-neprilysin inhibitor
PCSK9	Proprotein convertase subtilisin/kexin type 9
BM	Bone Marrow
LDL	Low-density lipoprotein
LCZ696	Combination of sacubitril and valsartan
EPCs	Endothelial Progenitor Cells
MSc	Mesenchyme Stem cells
MI	Acute myocardial infarction
CSCs	Cardiac Stem Cells

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Chapter 6

Concepts and Recent Advances on Biopolymers for Biomedical applications: Special Mention to the PHAs Family

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Abstract

Not only in the biomedical field but also in other applications, synthetic polymers are gradually being replaced by biodegradable materials, especially by those derived from natural resources. In this regard, many types of natural polymer or biopolymers have been developed to satisfy the ever-increasing application requirements. Since the demand for biomedical materials grows, significant attention is being given to tailor the structure, properties, and function of biopolymers to fulfill the requirements for applying them in biomedicine. Due to their inherent material properties, biopolymers are an appealing alternative to the synthetic polymers in the biomedical field. So far, a considerable number of natural polymers have been studied in detail regarding their suitability for applications in tissue engineering, wound-healing, bone regeneration, and drug delivery. Most of these biopolymers can be classified in the polyester, protein, polysaccharide, lipid and polyphenol families. In this chapter, the importance of biopolymers in the biomedicine is evidenced, and the main and most recent advances of the principal natural polymers used in this field are briefly reviewed, paying special attention to the natural biopolyesters, the PHAs family.

1. Introduction

Biomedicine is the theoretical branch of medicine that applies the principles of biology, biochemistry, and biophysics for the understanding of medical research and its practice. On one hand, an emerging area in biomedicine is that of biomimetic materials and systems. On the other hand, there is an imminent need for developing new materials for specific purposes in particular medical fields.

The main objective of implantable devices and biomedical structures is to mimic a body's system and/or to replace a damaged organ in order to maintain normal body functions. The three main families of materials, metals, ceramics, and polymers, have been applied to this purpose. However, they may present some disadvantages like immunological rejection by the body [1]. Especially, synthetic polymers may present concerns about their biodegradation products since they can lead to an undesirable immunogenic response [2, 3]. In general, it is difficult to mimic living systems and satisfy the growing biomedical needs with conventional synthetic materials alone. In some cases, the combination of both synthetic and natural materials can be a solution [4-7]. Nevertheless, biopolymers have been highlighted among the traditionally used materials and have been established as a promising class of biomaterials with a wide range of applications in biomedicine. Since they are produced by living organisms, biopolymers show unique properties such as degradability and biocompatibility, which provide them with advantages over other material families. They represent a solution for many biomedical applications due to the combination of their inherent properties, including great versatility and processability, biocompatibility, biodegradability, bio/absorbability and absence of cytotoxicity, all of which are essential properties that a material used for medical applications should possess. Thus, several studies using biopolymers as biomimetic materials are frequently found in the literature. For instance, Ochetta *et al.* [8] mimicked a fibrosis-like environment by embedded cardiac fibroblasts in a 3D fibrin-hydrogels. Bazrafshn *et al.* [9] recently reviewed the use of chitosan to mimic some body fibrous assemblies. Another recently used biopolymer to mimic the carbohydrate moieties of mammalian glycosaminoglycans is a sulfated polysaccharide found in the cell walls of the brown algae [10]. Examples of how biopolymers can be used in specific situations in biomedicine are the preparation of natural biocomposites. With the aim of reducing drug consumption, Ye *et al.* [11], prepared a biocomposite based on porous chitosan with silver nanoparticles that promoted wound healing and showed good antimicrobial activity and biocompatibility. Sharabi *et al.* [12] recently dressed one of the challenges of future research for the replacement or repair of the degenerated intervertebral disc. They developed a complex 3D biocomposite of long collagen fibers embedded in alginate hydrogel, which mimics the form of annulus fibrous lamellar. The mechanical behavior was found to reproduce the natural stress-strain behavior.

Since there is a continuing development and design of new systems involving biopolymers

for biomedical applications, the focus of this chapter is to provide a brief overview of the more recent advances in the application of the main biopolymers used in medicine, with emphasis on the of natural biopolyesters, the PHAs.

2. Biopolymer Recent Market and Environmental Aspects

We are now well aware of the environmental problems related to the huge quantities of wastes produced by human activity, especially in regards to plastic. Fossil-based polymers correspond, in general, to non-biodegradable materials, which leads to two principal problems: the accumulation of waste in natural environments, including the sea with negative effects on marine fauna through plastic ingestion, and the leaching of plastic products with the potential to transfer chemicals to human beings and wildlife [13]. Despite of that, the global production of plastics is increasing every year (according to Consumer News and Business Channel, more than 9 billion tons of plastic have been produced worldwide since the 1950s, of which 9% was recycled, 12% was incinerated and 79% was built up in landfills or disposed indiscriminately). As a result, there is a growing realization that organic matter from biological origins, with mainly a polymeric structure, can be a solution. Thus, there is a need to continue developing biotechnological processes to achieve large scale production of these natural occurring polymers. However, one of the major remaining concerns is the high production costs that present biopolymers from being economically competitive. Nonetheless, this market is continuously growing, and sophisticated biopolymers are emerging along with innovative applications in different fields, including that of biomedicine, and other new products. According to the latest market data collected by European Bioplastics in cooperation with the research institute Nova-Institute, global bioplastics production capacity is set to increase from around 2.11 million tonnes in 2018 to approximately 2.62 million tonnes in 2023. However, the annual capacity growth rate for bio-based polymers has been slowed down sharply since 2015 (reduced more than half) . This lower annual growth rate is mainly caused by the decrease of oil prices, low political support, a slower than expected growth of the capacity utilization rate and the populist debates about using food crops for industry use [14]. It is believed that, during the next few decades, the demand for these products will rapidly increase and they will be widely used in a broader range of applications.

In the late 1980s and early 1990s, innovative biopolymers were introduced to the market for the first time, and were mainly based on starch and polyhydroxyalkanoates (PHAs) produced by fermentation. These biodegradable first-generation biopolymers did not successfully become established in the market, mostly due to their yet unknown material properties, unfavorable political and economic circumstances, and a lack of political will [15]. In recent years, improved second-generation biopolymers have been developed almost exclusively as degradable and compostable materials for the packaging, agriculture and gardening sectors [15]. The trend among the third-generation biopolymer materials is away from degradability and instead

towards resistance (15). In parallel, the use of biopolymers from different origins has been investigated for many years for pharmaceutical and biomedical applications. This has resulted in a multitude of healthcare products on the market that is biopolymer-based. Nowadays, biopolymer production for biomedical applications only corresponds to approximately 1% of the annual polymer production. However, an increase of 19% is expected for 2020 compared to 2017. Among other natural polymers such as dextran, xanthan gum, and pullulanin, polylactic acid (PLA) and PHAs are the most recognizes ones for contributing to this increase [16].

3. Biopolymer Definition, Main Properties and Classification

Natural polymers or biopolymers may be defined as naturally-occurring polymeric macromolecules synthesized during the life cycles of plants, animals, bacteria or fungi [17, 18]. Since they are generated from renewable sources and their structural backbone is composed of oxygen and nitrogen atoms, they are easily biodegradable [19]. Biodegradation converts them into CO_2, water, biomass, humid matter, and other natural substances [20], making them harmless and non-toxic for the human body. As a result of their suitable properties such as good biocompatibility, biodegradability, and non-toxicity combined with versatile mechanical properties, there has been a growing demand for biomedical biopolymers in the last years, as well as an increase of their number and class [17]. Therefore, there are several classifications for biopolymeric materials. Usually, they are divided according to their repeating monomeric units in polynucleotides (DNA and RNA which are formed by nucleotide monomers), polypeptides (amino acids are their monomeric units) and polysaccharides (different carbohydrates structures) [17, 21]. They can be classified by their origin, depending on the synthesis and on the sources: from biomass (polysaccharides, protein and lipids, from animal or plants), from microbial production (PHA), from chemical synthesis using monomers obtained from agro-resources (such as PLA), and polymers whose monomers and polymers are both obtained by chemical synthesis from fossil resources (such as polycaprolactones, polyesteramides, aliphatic and aromatic co-polyesters). Biopolymers obtained from non-renewable resources are also included [22, 23]. The United States Congress Office of Technology Assessment classifies them into nucleic acids, proteins, polysaccharides, polyhydroxyalkanoates and polyphenols [17]. Their source origin classifies them in natural or semi-synthetic and based on their applications they can be bioplastics, biosurfactant, biodetergent, bioadhesive, or biofloculant [18]. In this chapter, a biopolymer classification based on the backbone of the polymer chain is presented (**Figure 1**). Special attention must be given to poly(lactic acid) (PLA), which in several cases is considered as a synthetic polymer. The production of PLA is based on the production of the lactide monomer from lactic acid, which is produced by the fermentation of agricultural source corn [24]. Then, high molecular mass PLA is produced by ring-opening polymerization of the lactide. In our classification, we consider PLA as a biopolymer since it is made from renewable resources.

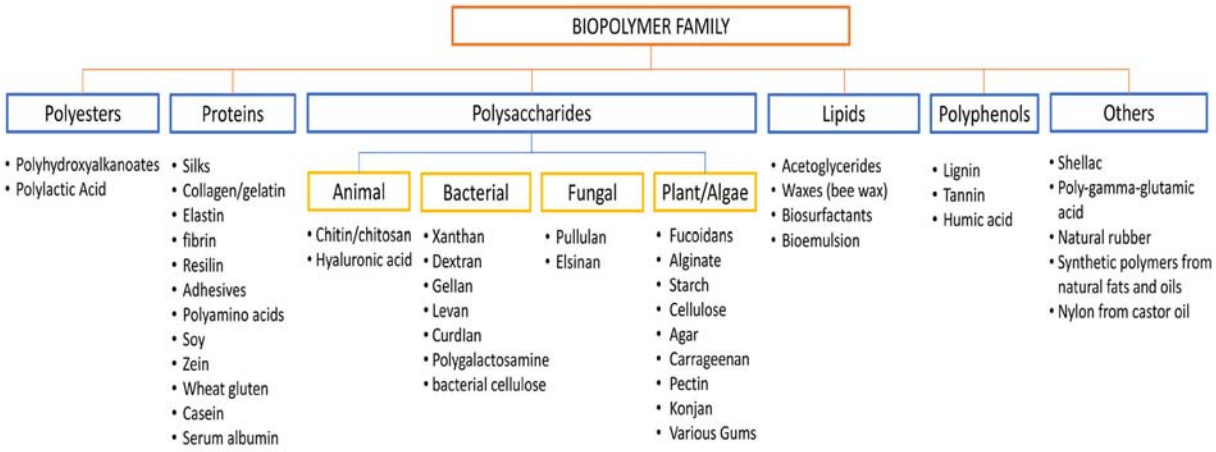

Figure 1: Biopolymer backbone-based classifications.

4. Most Recent Advances of the Main Biopolymer used in Biomedicine

4.1. Polyhydroxyalkanoates (PHAs)

PHAs represent a family of biopolyesters synthesized by several microorganisms. They are intracellular storages of carbon and energy, accumulated in the shape of granules [25]. They are produced when nutrients such as nitrogen, phosphate or oxygen are depleted, and there is an excess of carbon source. Under these conditions, microorganisms can divert the usual carbon flux (conversion of acetyl-CoA in the tricarboxylic acid cycle to create energy and metabolites for biomass formation) towards the synthesis of PHA [26,27]. PHAs can be produced by biotechnological processes *via* bacterial fermentation. *Cupriavidus necator* is the most extensively studied bacterial strain for PHA production on an industrial scale. *Azahydromonas lata* (formerly known as *Alcaligenes latus*), *Azotobacter sp.* and recombinant *Escherichia coli*, are among the PHA-producer bacteria, but to a lesser extent [28,29]. Especially, extremophile bacteria such as halophiles or thermophiles are of great interest in the production of PHAs [30]. Gram-negative halophile PHA-producers such as *Haloferax mediterannei*, *Halomonas campaniensis LS21, Haloarcula marismortui, Halomonas TD0, Bacillus megaterium* uyuni S29 have been reported for their high PHAs production. The strain *Chelatococcus sp*. is an example of a thermophile bacteria that is also studied for its ability to synthesis biopolymer [30]. Several microorganisms secrete extracellular PHA-depolymerases to degrade the biopolymer into oligomers and monomers, so that they can consumed these degradation products as nutrients [31]. This is the reason why these kinds of polymers has the inherent property of being biodegradable.

Chemically, PHAs are linear biopolymers composed of hydroxyalkanoate units (HA) as the basic structure (**Figure 2**). PHAs are biocompatible, biodegradable and non-toxic, and their members differ in their structure and mechanical properties, depending on the producing microorganism, the conditions of biosynthesis, and the type of carbon source used in the production process [32-34]. Poly-3-hydroxybutyrate (PHB) is the simplest and most commonly

produced PHA. It is a linear, unbranched homopolymer consisting of (R)-3-hydroxybutyric acid (HB) units [35]. Because of its competing thermoplastic and mechanical properties, which are similar to those of petroleum-derived plastics such as polypropylene, it is gaining interest as a substitute for these synthetic polymers [30,35]. Besides HB, microorganisms can incorporate up to 60 different types of monomers in their inner storage (**Figure 2**). For instance, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), a PHB copolymer, can be produced by adding valeric acid to the fermentation medium [36]. PHBV is characterized as less crystalline and more flexible than the PHB itself [37]. These properties vary according to the hydroxyvalerate content in the structure: a higher hydroxyvalerate monomer number leads to a lower crystallinity and to greater flexibility, strength, and elongation at break [34]. Another member of the PHA family is poly-4-hydroxybutyrate (P4HB), a resorbable, thermoplastic homopolyester with a linear chain structure of 4-hydroxybutyrate (4HB) monomers. It can be produced by using sodium 4HB as a precursor for its synthesis [38,39].

Globally, PHAs have gained considerable commercial interest in fields such as pharmaceuticals, veterinary scince, food packaging, agriculture, industry and especially in medicine because of their unique material properties [40]. Besides their non-toxic nature, biodegradability, and biocompatibility, they have antioxidant properties, optical activities, piezoelectric property, impermeability to gas, good resistance to ultraviolet, resistance to hydrolytic degradation, thermoprocessability, and stereospecificity [41]. In biomedicine, PHAs have been exploited in numerous forms when performing tissue engineering to repair the liver, bone, cartilage, heart tissues, cardiovascular tissues, bone marrow, and nerve conduits [34,38, 42,43]. Tissue engineering is an interdisciplinary field of research focused on the creation of vital tissues by a combination of biomaterials, cells, and bioactive molecules, aiming to repair damaged or diseased tissues and organs [44]. In this regard, P4HB has acquired importance in this field due to its unique set of properties and advantages: lower modulus, higher elongation and flexibility, and the ability to be oriented so as provide tensile strengths comparable or superior to existing resorbable synthetic polymers, such as poly(glycolic acid) (PGA) or PLA. kai *et al.* [38] review a list of applications where P4HB is used as heart valves, stents, and cardiovascular and pulmonary patches.

The degradation rate of a polymeric material is important for its exploitation as a biomaterial. Because PHAs have a biodegradable nature, they are used for absorbable sutures, surgical pins and staples, delayed drug release, and as drug carriers. Ali and Jamil [45] reported that PHB degrades more *in vitro* and in living mammalian cells than the other synthetic polymeric materials like PLA or poly(lactic-co-glycolic acid) (PLGA). The importance of degradability is reflected in the application of drug delivery systems. The latter consist of a material with an encapsulated active principle that is introduced inside the body to reach a located point for healing. It is by means of their biodegradation in the tissues of the host organism, that the

materials provide the liberation of bioactive substances. Drug discharges can be regulated over a determinate period of time, depending on the rate and degradation process of the material used. Recently, the use of PHAs in the form of coatings and micro- and nano-particles as resorbable matrices for controlled drug release has been reported [46-48]. **Figure 2 d** and **e** show Field Emission Scanning Electron Microscope (FESEM) images of these studies. These recent publications discuss the objective of overcoming implant-related infection and bacterial load on the implant surface. This promising strategy consists of using PHA biopolymers as drug carriers to control the release of antibiotic by the biopolymers degradation [41,48].

The versatile structure of PHA can be modified simply through physical blending and chemical alteration to improve its efficacy for medicinal use. PHAs have therefore been used in combination with other materials for fine-tuning their mechanical properties, and increasing their range of applications [49,50]. Recently, more sophisticated and complex PHAs have been developed. Examples include the production of PHBVHHx (consisting of a copolymer of HB, HV, and HHx) microspheres to serve as a carrier or scaffold to support cell growth for injectable purposes [51], and the poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate-co-3-hydroxydecanoate-co-3-hydroxydodecanoate) being used as a scaffold for tissue engineering [52].

Although the major obstacle for the broad commercial utilization of PHAs is that their production costs are still higher than synthetic plastics, PHAs show several advantages when compared with other synthetic polyesters such as PLA, PGA, and their copolymers, polycaprolactone (PCL), and PLGA, all of which are examples of biodegradable polymers used in biomedicine [53,54]. The first advantage is that the biopolymer production *via* fermentation prevents the presence of toxic products in the synthetic polymerization process. Second, the hydrolytic degradation of PHB leads to obtaining monomer D-3-hydroxybutyric acid, which is a common blood constituent (a ketone produced by the liver from fatty acids, ketogenesis). Third, the use of extremophile bacteria for PHAs production not only enables their cultivation under drastically reduced or even absent sterility precautions, but also reduce factors affecting the production cost of these biopolymers, the sterilization process [30,48]. Fourth, they can be produced from renewable resources, low cost raw and/or waste materials and this allows their production to be pollution-free and independent of the oil industry [28,29,52,55].

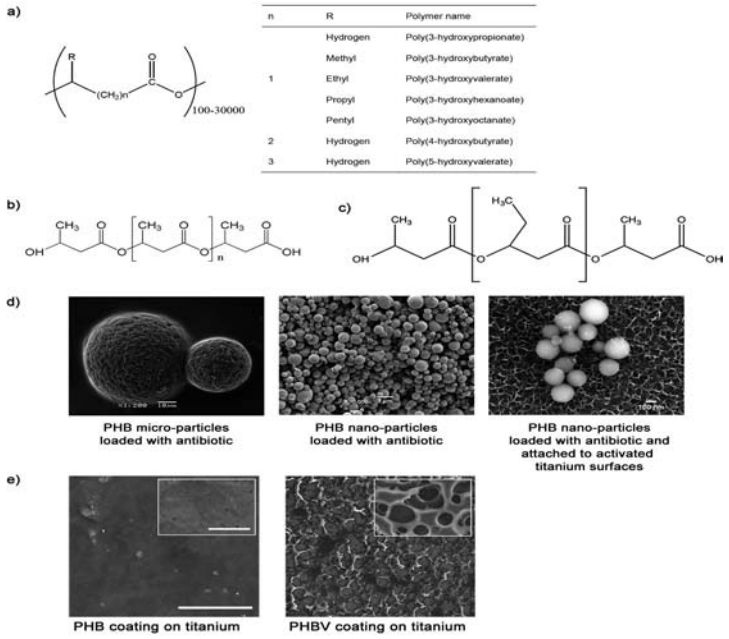

Figure 2: PHAs Chemical Structure and Application as Drug Delivery Systems: (a) PHAs are biopolyesters with hydroxyl and the carboxylic groups of the hydroxyalkanoic acids linked together via oxoester bonds. (b) PHB chemical structure. (c) PHVB chemical structure. (d) FESEM images of antibiotic-loaded PHB micro- and nano-particles for drug delivery systems [47]. FESEM images of antibiotic-loaded PHB nano-particles covalent attached on activated titanium surface [48]. (e) FESEM images of PHB and PHBV coatings loaded with antibiotic. The scale bar corresponds to 300 μm. The inset images correspond to higher magnifications micrographs. Scale bar corresponds to 40 μm [41].

4.2. Proteins: Collagen

A variety of proteins and protein-derived products (polypeptides) have been used and characterized for their use in medical and pharmaceutical applications. Protein-based matrices such as gelatin, albumin, elastin, casein, collagen, corn protein, and whey protein have been applied in biomedicine to form micro- or nano-spheres, hydrogels, films, and scaffolds [56, 57]. Among all these proteins, collagen is highlighted as one of the most abundant biopolymers within biomimetic materials and it is widely used in different areas of biomedicine.

The word collagen has a Greek origin and can be divided into “kola” and “gen”, which means gum and producing [58]. According to the Protein Data Bank, it is the most abundant fibrillar protein, and is available in the extracellular matrices of many connective tissues of mammals including skin, joints, cartilage, teeth (collagen joined to mineral crystals), tendon, bones, and others [58]. This structural protein comprises about 25-35% of the whole-body protein content, and its main function is to provide mechanical stability, strength, and elasticity to native tissue [9]. Since the discovery of collagen II by Miller and Matukas (1969) [59], 29 new collagen types have been found [60]. Various types of collagen, their tissue distribution and functions are widely described in the literature. Recently Muthukumar *et al.* [61], Lin *et al.* [60] and others [58] summarized this information. Among all collagen types, type I forms over 90% of the collagen of the body [9]. The structure of the collagen types can be grouped into fibrils, networks, beaded filaments, anchoring fibrils, and fibril-associated collagen with interrupted triple helices. These types of fibrils are the most common form, distributed in

most connective tissues [60]. **Figure 3** from Lin *et al.* (2019) show the fibrillar structure of collagen, from the proteins to the collagen fibers.

Collagen sources include bovine, porcine, and human origin, with bovine and porcine being the most commercialized. However, these together with other collagen sources used such as chicken neck (type I, II, III and V), kangaroo tail, rat-tail tendon, bird's beak, equine skin, cartilage and flexor (type I and type II), alligator bones and skins, sheepskin, frog skin, and so on, they are associated with the risk of transference of zoonotic diseases [58,62]. Recently, marine collagen, extracted from various marine sources (predominantly scale and skin fish) has emerged as the most appropriate alternative [63,64].

Due to its excellent properties, such as low immunogenicity, biodegradable, biocompatibility, hydrophilicity, easy processing, and weak antigenicity, collagen has become the primary resource of protein in medical applications [65,66]. However, collagen suffers from poor physical and chemical properties such as mechanical strength, thermostability, and resistance to enzymes [66]. Due to the extraction process, its mechanical properties and stability are lesser than those in its natural state. This seriously limits its potential in biotechnological applications. Consequently, crosslinking is a wide recognised solution for the improvement of its properties [67]. Exogenous crosslinks have been used to modify the molecular structure of collagen to minimize degradation and enhance mechanical stability [67]. There are different crosslinking methods, including physical, chemical and biochemical modifications. Physical crosslinking is carried out *via* UV or gamma radiation. For chemical modification - the most effective and most widely used crosslinking method for collagen - glutaraldehyde, isocyanates, hexamethylene diisocyanate, polyepoxy compounds, as well as plant extracts or inorganic crosslinking agents are the most utilized. Of recent, low toxic chemical crosslinking agents based on traditional biomasses such as dialdehyde cellulose or oxidized starch, are also employed. Enzymatic modification with oxidoreductases, transferases, and hydrolases is known as biochemical crosslinking [66, 67]. In this regard, there are however new studies that discuss the improvement of the mechanical properties of collagen by other alternatives. For instance, Rieu *et al.* [68] by novel process for collagen production, developed a collagen-only, non-cross-linked scaffolds with uncommon mechanical properties which they applied to 3D cell culture. As well, the blend of collagen with other biomaterials and biopolymers is another alternative to prepare collagen-based biocomposites with more suitable physical and mechanical properties [69].

The most relevant and advanced applications of collagen in biomedicine are: (1) Shields in ophthalmology [70-72], (2) sponges for burns and wounds [73-76], (3) mini-pellets for protein and drug delivery [56], (4) controlling material for transdermal delivery [77,78], (5) nanoparticles for gene delivery [79], (6) drug/gene delivery formulations for tissue healing, used in the form of film [80,81], sheet [82], disc or scaffolds [83], (7) 3D scaffold or gels

for cell embedding (68, 84-88), (8) organoids or neo-organs for gene therapy [89], (9) tissue engineering including skin replacement, bone substitutes, and as artificial blood vessels and valves [61,67].

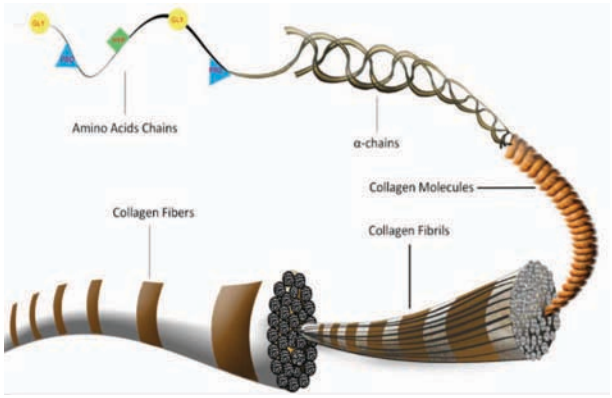

Figure 3: Collagen Structure [60]: Collagen is composed of specific amino acids including glycine, proline, hydroxyproline as the smallest units. According to particular alignment with other amino acids, it becomes *peptide chains* (α1, α 2, β chain). Three of the same or different peptide chains tangle together form triple helices. This is called *collagen molecule*. Many triple helices crosslinked together form *collagen fibrils*. Several of collagen fibrils crosslink together to become *collagen fibers*.

4.3. Polysaccharides

Natural polysaccharides have been recognized and applied as viable candidates for various biomedical, pharmacological and biotechnological applications. Within these fields, saccharides, oligosaccharides, and polysaccharides are used for bioactive therapies, diagnosis, controlled drug release, gene therapy, cell-encapsulation, tissue engineering, and medical devices [90]. They are of special interest due to their high abundance, good biological performance, structural similarity to the extracellular matrix, and degradability by enzymes present in the body [91]. Polysaccharides can be obtained from a variety of sources including human and animal, bacterial, fungal or vegetal origins (**Figure 1**).

4.3.1. Polysaccharides from Animal Source: Chitosan

The exploitation of the sea as a renewable source of biocompounds provides a positive step in the development of new systems and devices for biomedical applications. Marine polysaccharides are among the most abundant materials in the seas. While alginate, carrageenan and fucoidan polysaccharides are extracted from algae, chitosan and hyaluronan can be obtained from marine animal sources. They show important biological properties like biocompatibility, biodegradability, and anti-inflammatory activity, as well as adhesive and antimicrobial actions. [92]. Among them, chitosan and its oligosaccharides have received considerable attention due to their biological activities and properties in commercial applications [93]. Chitosan is a molecule with a carbohydrate backbone structure similar to cellulose, consisting of two types of repeating units: N-acetyl-D-glucosamine and D-glucosamine monosaccharides, bonded together with a (1-4)-β-glycosidic linkage (**Figure 4**) [94-96]. It is a biopolyaminosaccharide natural polymer that is obtained by treating the chitin *via* alkaline deacetylation [96]. Chitin

was first isolated and characterized from mushrooms by the French chemist Henri Braconnot in 1811 [97]. Except for celluloses, chitosan is the most abundant polysaccharide in nature. It is the main component of the exoskeleton of crustaceans and insects, and also occurs in nematodes and in the cell wall of yeast and fungi (**Figure 4**) [17] [92]. Until recently, only marine sources (shrimp, prawn, crab) have been used to provide the starting chitin. Lately, new commercial chitosans, better characterized by manufacturers and with enhanced safety characteristics for certain pharmaceutical, cosmetic, and biomedical applications have been produced at lower costs [98]. Chitosan is one of the marine polysaccharides most widely used and studied for biomedical applications, not only because it has revealed some therapeutic activity such as lowering of cholesterol, wound healing, antiulcer, and antimicrobial effects [96], but also due to its non-toxicity (it has been approved by the US Food and Drug Administration), its biodegradability, and bacteriostatic and fungicidal characters [94,96,99]. Furthermore, it shows advantages in regards to its special used as drug carrier, and thus it has been extensively exploited in the preparation of micro-/nano-particles, beads, and capsules for controlled drug delivery systems [96,100-102]. Ahmed *et al.* [96] describe some of its advantages that make chitosan the appealing biopolymer for the development of polymeric particles: its mucoadhesive nature (which increases the time of attachment at the absorption site), the easy availability of free amino groups (for cross-linking),the ease of fabrication of polymeric particles without using hazardous solvents, the cationic nature that permits ionic cross-linking with multivalent anions, and its ability to control the release of an administered drug. Also, membranes, films and scaffolds **[**94,103**]** of chitosan have benn developed for tissue engineering, regenerative medicine and therapy [100]. Recently, Bazrafshan *et al.* [9] reviewed the use of chitosan to mimic fibrous assemblies. Chitosan can be also mixed with other synthetic or natural polymers in order to help its processability and fine-tuning its properties [94].

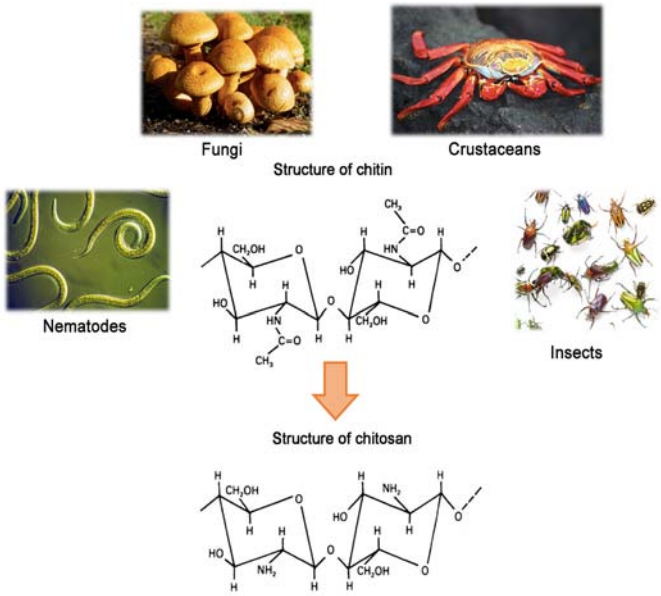

Figure 4: Structures of Chitin and Chitosan [95]: Chitin is obtained from different animal sources (nematodes, fungi, crustacean and insects) , especially from the demineralization and deproteinization crustacean shells and insect exo-squeleton. Then, chitosan is obtained by removing the acetyl groups (CH3-CO-) of chitin. This process, called deacetylation, releases amine groups (NH2) and gives chitosan its cationic characteristic.

4.3.2. Polysaccharides from Bacteria Source

Most microorganisms are able to secrete exopolysaccharides (EPS's) naturally into the extracellular environment. They are high molecular mass biopolymers, showing extreme diversity in terms of chemical structure and composition. EPS's tend to be bioactive, depending on their backbones, chain length, and substitution [104]. The use of these bacterial EPS's in medical applications started with the first clinical trials on dextran solutions as plasma expanders in the middle of the 20th century [105]. Later, other bacterial EPS's such as xanthan or pullulan were used in medicine as pharmaceutical excipients (as suspension stabilizers and in capsules and oral care products, respectively). A number of other EPS's has been added to the list, counting with alginates used as anti-reflux, dental impressions, or as matrixes for tablets, hyaluronic acid (also called hyaluronan) and derivatives used in surgery, arthritis treatment, or wound healing, and bacterial cellulose applied in wound dressings or scaffolds for tissue engineering [105,106]. The following table summarises EPS's naturally produced by different microorganisms, and the most recent advances where they are applied in the biomedical field.

The use of microbes to produce EPS's shows several advantages over plant- or macro algae-derived products that make them more suitable for industrial and commercial use. The production time (the obtaining of EPS's from bacteria takes only days compared to months from plant-based products), the surface required (there is no land needed for cultivation), the controlled production with defined and reproducible parameters, and the high quality of the final product are some of these advantages [105,106]. However, the production cost is still one of bacterial EPS disadvantages, since the expenses are directly related to the cost of the substrate, required for microbial growth, as well as the cost of bioreactors to grow microorganisms in large quantities [106]. Even so, the possibility of finding new bacterial polysaccharides with bioactive properties and potential applications in the fields of pharmaceutics, cosmetics, and in biomedicine is still being investigated [107,108].

4.3.3. Polysaccharides from Fungal Source

There is growing interests in polysaccharides being isolated from mushrooms, which are recognized as safe and effective natural antioxidants. For a long time, mushrooms have attracted significant interest as traditional food and medicine. They are also used as functional health promotors because of they are biochemically composed of significant amounts of carbohydrates, lipids, proteins, enzymes, minerals, and vitamins. Polysaccharides such as pullulan, elsinan, and yeast glucans, which are among the most important active components of mushrooms, have been reported to possess broad-ranging and potentially valuable pharmacological properties in biochemical and medicinal areas, including anti-tumor, anti-inflammatory, immunomodulatory, and in particular, antioxidant activities.

Table 1: Application of some ESP in biomedicine

ESP	MICROORGANISM	APPLICATION	REFERENCE
Xanthan Gum	bacterium *Xanthomonas campestris*	Intra-abdominal adhesion, high thickening capacity, emulsifying, film forming, release control agent	[109, 110]
Gellan	*Bacterium Sphingomonas paucimobilis* (formerly *Pseudomonas elodea*)	Excipient in oral, ophthalmic and nasal drug formulations, gelling/thickening agents, drug release, scaffolds for bone tissue engineering applications, cell encapsulation.	[105, 111-115]
Dextran	Bacterium *Leuconostoc mesenteroides*	Molecule-carrier or drug delivery system, plasma volume expander, peripheral flow enhancer, antithrombolytic agent and for the rheological improvement for artificial tears	[105, 116, 117]
Alginate	Several bacteria strains *Azotobacter vinelandii, Pseudomonas aeruginosa*	Controlled drug release, encapsulation, scaffolds in ligaments, tissue engineering and in dentistry for the preparation of forms in the presence of slow-release calcium salt, cell microencapsulation	[118-120]
Hyaluronic acid/ hyaluronan	Bacteria *Streptococcus equisimilis/zooepidemicus, Bacillus subtilis*	Gelling/thickening agents, skin regenerating, collagen and elastin stimulating efficacy, drug release for treating tumor cells, skin regenerating and collagen stimulating efficacy	[112, 120-123]
Bacterial cellulose	Aerobic bacteria, belonging to the genus *Acetobacter (*primarily by *Gluconacetobacter xylinum)*	Artificial skin, artificial blood vessels and microvessels, wound dressing, implants and scaffolds for tissue engineering, carriers for drug delivery, wound-dressing materials	[124-127]
Levan	Different bacteria, *Bacillus polymyxa* PTCC1020, *Bacillus subtilis, Aerobacter levanicum, Erwinia herbicola, Streptococcus salivarius* and *Zymomonas mobilis*	Thickeners and encapsulating agents. film agent, a carrier for drug delivery systems, an anti-inflammatory compound or its potential use for functional food as prebiotic.	[128-130]
Polygalactosamine	Bacterium *Paecilomyces sp*	Growth inhibitor of some tumor cells. With Chitosan microspheres for drug delivery system	[131]
CurdIan	*Agrobacterium* species	Inhibit tumors, anti-HIV effect, tablets and gels for drug delivery	[132]

Among these bipolymers, pullulan is a natural linear homo-polysaccharide obtained from the polymorphic fungus *Aureobasidium pullulans*. It consists of three glucose units attached by α(1→4) glycosidic linkages, which are attached to each other by α(1→6) glycosidic linkages **[**16,133,134**]**. There is extensive work to improve the production of pullulan as well as its yield by changing fermentation parameters or the substrate used in order to improve their economic viability [16,135-138]. Pullulan exhibits unique physicochemical properties such as high water solubility and biodegradability. This is due to the coexistence of different glycosidic bonds [133,138]. Pullulan is used as a stabilizer, an adhesive, and a coating or packaging material in the food industry. Because of its inherent non-toxic, non-immunogenic, and biodegradable characters [133], it also offers a wide range of potential applications in biomedicine such as targeted drug/gene imaging and tissue engineering. In particular, pullulan has been used as hydrogels for tissue engineering. Wong *et al.* [139] demonstrated that pullulan hydrogels are an effective cell delivery system, and improve mesenchymal stem cell survival and engraftment in high-oxidative-stress environments. From pullulan, Autissier *et al.* [140]

prepared and evaluated a novel biomaterial for vascular engineering, consisting of pullulan gels with water-content higher than 90%. Pullulan-collagen composite hydrogel matrices were fabricated by Wong *et al.* [141], resulting in a structured yet soft scaffold for skin engineering. More recently, the novel topical film prepared with verniciflua extract-loaded pullulan hydrogel was synthesized for atopic dermatitis treatment [142]. Similarly, Zhang *et al.* [133] developed a gelatin hydrogel with oxidized-pullulan. which gave extraordinarily high strength and mechanical enhancement to the hydrogel. Hydrogels-based scaffolds [143], microbeads [144] and composites [141,145] were also created with this fungal polysaccharide.

4.3.4. Polysaccharides from Plant Source: Starch

A substantial amount of research indicates that polysaccharides derived from herbs can be effectively used in many applications and have diverse therapeutic properties such as antioxidant, antitumor and immunostimulatory activities, and the effect of promoting wound healing [146]. Starch is one of the most abundant polysaccharides from plant origins, and has been used in food applications such as a thickening, binding, sweetening, and also as emulsifying agents [147]. It is mainly obtained from cereals and tubers. Chemically, starch is a polymeric carbohydrate composed of glucose units linked together, comprising two types of α-glucan: linear amylose (poly-α-1,4-D-glucopyranoside) and branched amylopectin (poly-α-1,4-D-glucopyranoside and a-1,6-D-glucopyranoside). Therefore, it is stablished as a heterogeneous material. This polysaccharide is produced from agricultural plants, mainly potatoes, rice, maize, and wheat. Depending on the botanical source, the percentage of each glucan type varies, as well as the whole morphology and molecular structure. Starch is a water-soluble biopolymer that produces viscous dispersions, solutions, or gels at low concentrations [13]. Structurally, native starch occurs mostly in the form of semicrystalline granules, with a complex hierarchical structure. These granules are generally composed of an amorphous bulk core surrounded by altered concentric semicrystalline and amorphous growth rings. Its availability of hydroxyl groups makes it tremendously hydrophilic and easy to chemically react (esterification, oxidation, etherification, and cross-linking) [148]. **Figure 2a** shows a scheme from Wang *et al.* [149] of the starch structure.

Due to its extensive availability, low cost and total composability without generating any hazardous residues, starch is used for a number of biomedical applications such as tissue engineering, wound healing, bone regeneration, and drug delivery, and it has also been used for adhesion, proliferation, differentiation, and regeneration of cells [13,44]. The employment of starch for biomedical functions is also appealing due to its similarity to the native cellular environment [44]. In order to enable applications in tissue engineering, starch has been manipulated to improve some of its properties such as its mechanical properties and moisture sensitivity [44]. Starch alone is inadequate to develop scaffolds. However, its mechanical stability can be improved to convert the material to an appropriate option. For

instance, Waghmare *et al.* [44] developed starch-based nanofibrous scaffolds using polyvinyl alcohol (a non-toxic, water-soluble, biocompatible, synthetic polymer) as the plasticizer and glutaraldehyde as a crosslinking agent for application in wound healing. The evaluation of the nanofibrous scaffolds in cellular assays demonstrated their non-toxicity and their ability to promote cellular proliferation. The strategy of employing starch as matrix not only reduced production costs, but also endowed the products with the features of biodegradation, biocompatibility and specific interactions with biological systems. Among other biopolymers such as alginate, gelatin, and collagen, starch is also used for bone substitution to fabricate scaffolds for bone tissue engineering. Aidun *et al.* [148] recently reported the fabrication of a bioactive porous scaffold of starch-siloxane for bone regeneration by cross-linking with 3-glycidoxypropyltrimethoxysilane as a biocompatible and hydrophobic material. The ability of the growth and proliferation of bone marrow mesenchyme stem cells on the constructs confirmed the suitability of these scaffolds for bone tissue engineering applications. **Figure 5** shows the surface topography of the starch-siloxane scaffolds. In regards to drug delivery system, starch has been used as particles and hydrogels. The group of Shi *et al.* [150] synthesized starch-based fluorescent organic nanoparticles for biomedical applications, while Gholamali *et al.* [151] recently developed a novel type of nanocomposite by combining copper oxide nanoparticles with oxidized starch hydrogels as a controlled drug delivery system (**Figure 5d**).

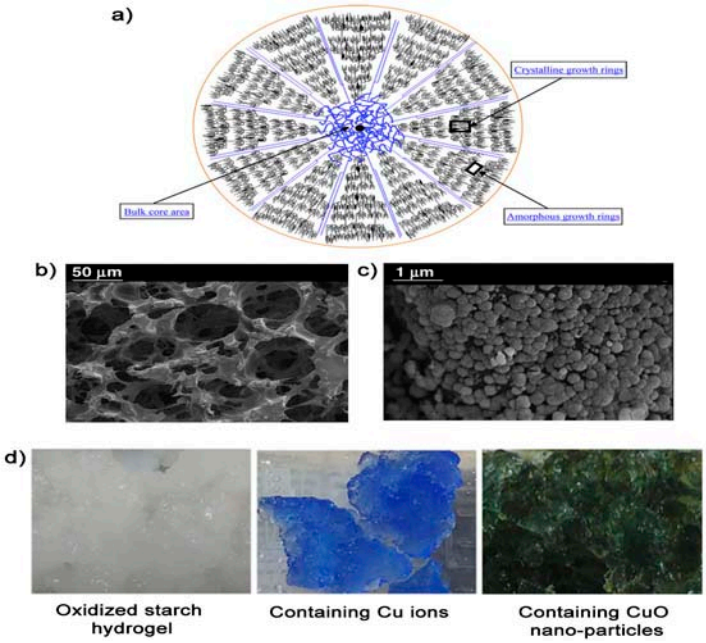

Figure 5: Inner Starch Structure: (a) Stylized model representing the distribution of amylose and amylopectin molecules. The blue lines in represent amylose molecules, and the black lines represent amylopectin molecules (149). FE-SEM images of (b) freeze-drying starch-siloxane scaffold, and (c) mineralized hydroxyapatite on the scaffolds [148]. (d) Photography's of oxidized starch hydrogels [151]. Copper ion (Cu) and copper oxide (CuO) nano-particles were incorporated into the hydrogel matrix.

4.4. Lipids: Biosurfactants and Bioemulsions

Microorganisms produce a variety of surface-active compounds (SAC), classified as biosurfactants and bioemulsifiers. The terms “Bioemulsifiers” (BE) and “Biosurfactants” (BS) are not interchangeable, and their definitions are based on their physico-chemical properties and physiological roles [152]. The natural SAC have become important products of biotechnology for industrial, pharmaceutical and biomedical applications [153,154]. As they are mostly produced on microbial cell surfaces or excreted extracellularly, they can be produced *via* fermentation using cheap agro-based substrates and other waste materials; unlike the synthetic surfactants, which are petroleum-derived. In general, they are amphiphilic compounds composed by both hydrophobic and hydrophilic groups that confer the ability to accumulate between fluid phases and reduce their interfacial tension [154,155]. These microbial SAC have different chemical structures, and surface properties, and are mainly classified according to their chemical composition, microbial origin, mode of action, molecular mass, and general physico-chemical properties [155].

4.4.1. Biosurfactants (BS)

BS are low-molecular-mass molecules microbial products, generally glycolipids, lipopeptides, and proteins with a lower surface and interfacial tensions between different phases [155]. The glycolipids (rhamnolipids, sophorolipids, trehalose lipids) consist of different sugars linked to β-hydroxy fatty acids, while lipopeptides (surfactin, iturin, fengycin) consist of cycloheptapeptides with amino acids linked to fatty acids of different chain lengths. These amphiphilic molecules are soluble in both polar and non-polar solvents [152]. The important features that biosurfactants have as compared to chemically synthesized surfactants are their biodegradability, bioavailability, lower toxicity, higher foaming, and high specific activity at extreme pH, temperature and salinity [156].

The best-studied glycolipids BS are rhamnolipids synthetized by several species including *Pseudomonas aeruginosa*. They are usually produced as a mixture of two or four species, by natural fermentation. They differ by the length of hydrophobic chains (from C8 to C12) some of which are unsaturated with one double bond (**Figure 6**) [157]. Rhamnolipids are biodegradable low toxic BS, with antimicrobial and anti-biofilm-formation properties [158, 159]. Therefore, rhamnolipids are used as a biofilm control agent to prevent medical device-related infections and to inhibit biofilm formation. They are also an anticancer agent, which inhibits the growth of many f the human cancer cell lines [160]. An example of the biomedical application of these biosurfactants is described in the recently published work of Jovanovic *et al.* (159) who used rhamnolipids to prevent adhesion and biofilm formation of *Candida albican.*

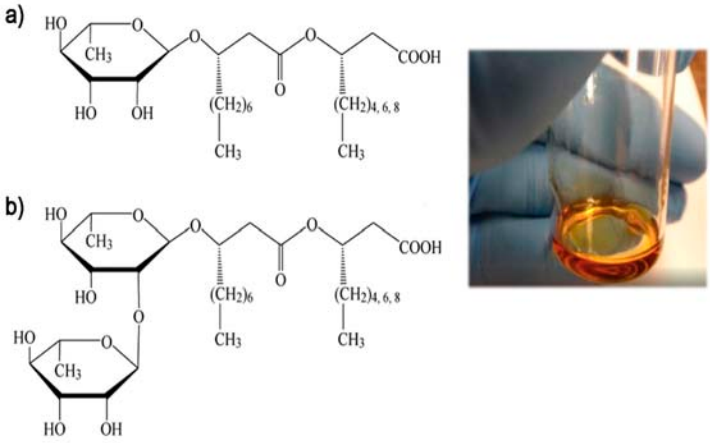

Figure 6: Structure of Rhamnolipids: Under typical growth conditions with Pseudomonas aeruginosa, two main rhamnolipids homologues are obtained: (a) monorhamnolipid (RL-1) and (b)dirham-nolipid (RL-2) [157]. Image of Rhamnolipids extract [3].

Most *Bacillus* species were found to be able to produce lipopeptides BS such as *Bacillus pumilus*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus licheniformis* [161]. Studies show that most of these *bacilli* can produce one type of lipopeptide, and just a few of them can produce two or the three types together [161]. In the case of surfactin, *Bacillus subtilis* K1 and *Bacillus siamensis* are known to extracellularly secrete it [152]. Surfactin is a cyclic lipopeptide that exhibits very good emulsification activity as well as excellent emulsion stability, and it has been found to be a better surface active agent in comparison to iturin and fengycin [156]. This BS is used in the biomedicine field as an antibacterial, antiviral [162], anti-tumoral, anti-coagulant agent, and shows broad-spectrum inhibition activities [152]. In a very recent study, the surfactin antibacterial activity against various Gram-positive and Gram-negative bacteria was confirmed [163]. An application of surfactin in biomedicine is seen in the recent work of Wang *et al.* [164]. They developed a novel "mosaic-type" nanoparticle system for selective drug release targeting hypoxic cancer cells, by assembling nanoparticles with surfactin. Another example is the study of Xing *et al.* [165], who used iturin together with surfactin in the form of enteric-coated insulin micro-particles for the oral drug delivery.

4.4.2. Bioemulsifiers (BE)

Several bacterial species from different genera produce extracellular polymeric emulsifiers composed of polysaccharides, proteins, lipopolysaccharides, lipo-proteins or complex mixtures of these biopolymers [152,153]. They are high molecular mass BE, which bind tightly to hydrocarbon surfaces and form stable emulsions by increasing kinetic stability in very low concentrations [152,155]. The first well-studied BE is Emulsan RAG-1 (1000 KDa) which is an extracellular poly-anionic BE produced by Acinetobacter calcoaceticus RAG-1 [155,166]. Yi et al. [167], recently used Emulsan RAG-1 to create oil in water-type nanoparticles loaded with pheophorbide to create a drug delivery system for treating tumor tissue. Another well-studied BE with potential environmental and biomedical applications is Alasan (45-230 KDa). It is produced by Acinetobacter radioresistens and is a complex union

between anionic polysaccharide and protein. In the case of Alasan, if the protein portion is damaged (by being digested by proteolytic enzymes), the BE turns into a thick polysaccharide, losing its emulsifying properties [155,166].

4.5. Polyphenols: Tannins and Lignins

Natural phenol-based polymers are widely represented in nature, and they include a variety of classes like tannins and lignins, which are the most prominent. Polyphenols are especially found in highly consumed foods: grape skin and seeds, seaweeds, wood and agro-wastes, primarily grape pomace and other by-products of fruit and coffee processing [168, 169]. Several phenolic polymers have been evaluated as biomaterial additives to favor cell growth and differentiation. Thanks to their antioxidant and antimicrobial properties [170], polyphenols have been found to stimulate bone formation, and mineralization, as well as stimulate the proliferation, differentiation, and the survival of osteoblasts [171]. They are able to counteract the inhibitory effects of reactive oxygen species (ROS) during the process of bone formation by osteoblastic cells [169]. Furthermore, polyphenols improve the performance of biomedical devices used in cardiovascular systems by improving the mechanical properties of grafted heart valves, enhancing microcirculation through the relaxation of the arterial walls, and improving capillary blood flow and pressure resistance [171].

The recently discovered phlorotannins are a peculiar class of tannins that are produced exclusively by marine brown seaweeds [172,173]. They show very especial properties such as antimicrobial, antioxidant, anticancer, radiation protection, anti-coagulant and other pharmacological activities [169,172]. Especially, they are effective in enhancing osteoblast differentiation and promote intracellular calcification [169]. In an application of these tannins, Douglas *et al.* [174] enriched mineralized gellan gum hydrogels with phlorotannins to endow antibacterial properties and promote mineralization with calcium phosphate uptake. More recently, Park *et al.* [6] fabricated a poly(vinyl alcohol) hydrogel for wound healing application, which showed an increase in cell attachment and proliferation when phlorotannins were added to the system. The study for hard tissue regeneration of Im *et al.* [175] demonstrated that their polycaprolactone scaffolds supplemented with collagen extracted from fish skin and phlorotannins exhibited marked calcium deposition and osteogenesis abilities compared to the ones without polyphenols supplements.

Lignocellulosic biomass is the most promising renewable carbon-containing source on Earth. Depending on the origin and species of the biomass, lignin consists of 20–35% of the lignocellulosic biomass. After it has been extracted, lignin can be modified through diverse chemical reactions [176]. The interest in lignin for biomedical applications lies in its specific antioxidant and antimicrobial activities. Lignin is utilized as a renewable macromolecular building block for the preparation of polymeric drug encapsulation and scaffold materials

[177,178]. For example, Kai *et al.* [179] created nanofibers of PLA-lignin copolymers further blended with poly(L-lactide) (PLLA) and demonstrated that the addition of lignin protects cells from oxidative stress conditions. Among the recent studies with lignin, Vinardell *et al.* [178)] reviewed some of their pharmacological activities for the treatment of diabetes and obesity control, along with other properties such as its antiviral, anti-coagulant and anti-emphysema, activities, and their application as nanoparticles for drug delivery. Figueiredo *et al.* [176] reviewed recent developments in the design and fabrication of lignin-based nanostructures for biomedical applications.

5. Conclusions

There is an increasing awareness of the danger of synthetic materials, and the negative environmental consequences that come with their excessive use. As a result, there is a growing motivation to use more natural resources or substitute synthetic materials by other ones with less environmental impact. Since synthetic polymers are now known to be a threat to the environment, natural polymers have come to play an important role in different areas of application. In the field of biomedicine, biopolymers show many advantages precisely because of their natural origin. Many biopolymers show common properties such as biocompatibility, biodegradability, and non-toxicity, which make them very appealing for their application not only in biomedicine but also in other fields like pharmacology and biotechnology. Due to their similarity to the native natural environment, their biopolymer functions show good biological performance, adaptability and adequate body reaction. Their mechanical properties are proving to be very versatile in many biopolymer families. Furthermore, there are now technological advances which can vary and tune their mechanical properties by chemical and physical treatment to make them exploitable in a greater range of applications. In most cases, the production cost is still one of the drawbacks that make biopolymers not yet competitive with other synthetic materials, making it relevant to continue investigating the production processes in order to economically optimize their efficiency.

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