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# Gene editing (CRISPR-Cas) technology and fisheries sector

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## Abstract

Considering the advantages of gene editing technologies, in recent years, emphasis has been given on three main techniques of gene editing i.e. zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the CRISPR/Cas9 RNA-guided endonuclease system. In all of these three technologies one thing is common i.e. the technology utilizes restriction enzymes to break down the double stranded DNA molecule at a targeted location with the help of another molecule of homologous binding protein or RNA, which is also called as a guide RNA molecule. This targeted breaking and repair of the DNA molecule as per our requirement is generally viewed as a great breakthrough in gene therapy methods. In fisheries sector, in order to promote aquaculture/mariculture activities, aquaculture industries are facing number of challenges, particularly in area of quality and demand of seed production, control of health and disease management, production of quality traits with phenotypically improved varieties, and strengthening of immune system. Several efforts are being made both at public and private sectors to develop scientific technologies to meet these challenges and substantial achievements have also been made. But still these challenges remained as major constraints in hampering the growth of this industry as per the demand and expectations. However, with the advent of now recently developed gene editing techniques, we may have to explore and evaluate the possibilities of applications of this technology as a long lasting solution in addressing at least some of the vital issues of the aquaculture industry particularly in those related to altering targeted gene structure in the species for positive impact.

**Keywords**: Gene editing, CRISPR-Cas, Biotechnological challenges, Fisheries sector

## Introduction

Fisheries and aquaculture sectors in India are vital sectors of food production, providing nutritional security not only to the weaker section of the society but also to other classes of communities irrespective whether rich or poor and also contributing substantially to the total agricultural exports in the country. India is the second largest fish producing country in the world with the current estimated fish production of 9.58 million metric tons [1]. With diverse resources ranging from deep seas to lakes, reservoirs, rivers, ponds, derelict water bodies, floodplains and more than 10% of the global biodiversity in terms of fish and shellfish species, the country has shown continuous and sustained increase in fish production since independence. Constituting about 6.3% of the global fish production, the sector contributes to 1.1% of the GDP and 5.15% of the agricultural GDP. The national mean production levels from still-water ponds has gone up from about 600 kg/hectare/year in 1974 to over 2900 kg/hectare/annum at present and several farmers are even demonstrating higher production levels of 8–12 tons/hectare/year. Fish and fish products have presently emerged as the largest group in agricultural exports of India, with 10.51 lakh tons in terms of quantity and Rs. 33,442 crores (5.14 b$) in value in 2014-15. This accounts for around 10% of the total exports of the country and nearly 20% of the agricultural exports. More than 50 different types of fish and shellfish products are exported to 75 countries around the world [1].

In order to boost the fish production and maintain the quality of the traits, definitely we may have to use modern scientific techniques. For past 4-5 decades, several efforts have been

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made in this direction and the success that has been achieved is also limited. There are number of biological issues of the aquaculture industry particularly in the area of disease control, biotic and abiotic stresses, increase in the growth and yield, impact of the environmental factors, etc. which are to be addressed using newer powerful technologies. In recent years, with the introduction of new genome editing tools such as Zinc-finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs) and more recently the clustered regularly inter-spaced short palindromic repeats (CRISPR)/ CRISPR- associated nuclease 9 (Cas9) systems, it has become possible to edit gene or knock out undesired part of the gene in different animal models [2]. The CRISPR/ Cas9 system has been introduced as a new class genome engineering tool even for those organisms where genome editing will be difficult. With this new promising technology we may have to think if we can alter the entire scenario of fisheries sector particularly that of aquaculture industry which is facing number of challenges while growing the aquatic animals in captive conditions. The CRIPSR-Cas technology can play an important role in devising molecular tools for accelerating reproduction, breeding and spawning, in disease control and therapeutics, in developing new traits of quality animals, in immune strengthening, and in achieving faster growth in captive environment.

The advantage of using this technology is not to alter or replace entire genome which otherwise happens in transgenic animals or genetically modified (GM) products, but to change or edit the targeted locations of the genome to create positive impact of the functional properties. This will result in the production of non-GM products which is more acceptable to the people and possibly may fit within the frame work of the Biotechnology Regulatory Authority of India. Therefore, products derived from gene editing could have a relevant socio- economic impact and public persecution compared to a GM product.

## Biotechnological challenges

World fish production has reached to approximately 158 million tons in the year 2014 according to the Food and Agriculture Organization (FAO) estimates [3]. Aquaculture sector is playing a vital role for increasing fish production all over the world. Almost one third of fish consumed today is produced from the aquaculture farming. For enhancing the production of aquaculture species, new scientific tools are being developed and used and the knowledge pertaining to the biotechnology is playing very important role and has a wide range of useful applications particularly in the areas of increasing growth and yield of commercial aquaculture species, for improving genetic traits with desired characteristics, strengthening immune system, fish health and disease management, boosting the nutritional value of aqua feed, restoring and protection of the environment, diversification of species, and conservation measure of the wild stocks. Some biotechnological tools are traditional and developed based on indigenous technical knowhow while others are more advanced based on the new discoveries of research in molecular biology and genetic engineering. In the field of genetic biotechnology, simple technique like hybridization to more advanced technique such as the transfer of specific genes between species to create Genetically Modified Organisms (GMOs) are being commonly used [4]. But now with the advent of new technique of gene editing, there is a possibility of creating new trait of species with desired properties without replacing the whole genome.

Biotechnology research as on today has the capability to address number of issues not only related to the aquaculture farming but also those concerning with the environment. In the semi-intensive and intensive fish culture systems, one of the major concerns is the source of protein used in the preparation of fish diet. The source of protein is generally from the fish meal as this contains high amount of animal protein. Fish meal manufacturing industries depends up on trash fish/low value fish as raw material for the preparation of fish meal. The processing cost of fish meal production is always high and not economical to the fish farmers. Therefore, there is a need to find out the alternate source of cheaper protein which can be used in the fish diet [4]. Another concern of fish meal production is continuous supply of raw material to the manufacturing units in the form of trash fish from the wild stock as per the demand of fish meal producers and also stability in the production. Due to an increase in wild fish catch all over the world, the world fish stocks are declining day by day resulting in the non-availability of continuous supply of trash fish and reduction in fish meal production. Moreover, the use of fish meal in the fish diet creates environmental problems in the fish farming system. The fish meal contains high amount of phosphorus which leaches out in the pond water that leads to the formation of algal bloom and eutrophication affecting the fish culture system [4]. As a result of these concerns with fish meal, researchers are using biotechnology to produce alternative plant-based protein source [5]. In an effort to find out the alternate source of protein, number of workers has diverted their focus of research to derive protein from the plant sources. Though plant proteins may address the problem of phosphorus content in the fish meal but there can be possibility of the presence of other ingredients in plant proteins which may create hurdles in the fish diet. It has been reported that there are number of anti-nutritional compounds present in plant proteins [4]. Such compounds one has to evaluate and find out for their elimination by intervention of biotechnological tools. Now the recent focus of research is on introducing some enzymes (phytase) in the fish diet which can act as anti-nutritional factors. The enzyme phytase can help fish to make the best use of the phosphorus available in plant protein based feed [4].

Madany et al. (1996) while working on trace metals concentrations in marine organisms reported that the farmed aquatic animals are very sensitive to the various environmental factors and will have to depend mainly on oxygen and other organic and inorganic compounds for their survival and growth. While inhabiting in such environment they also absorb and carry other wastes from nearby environment. There is a possibility of disease outbreak in this kind of environment which may result in mass mortality of fish [6]. Verschuere et al. (2000) reported that removal of pollutants from the aquatic environment can be done by using the technique of bioremediation. They further mentioned that either scavenging bacteria or novel probiotics can be developed and used for controlling in the aquaculture system [7]. Researchers are making continuous efforts to improve the genetic traits of the fish using various genetic engineering techniques. Efforts are also being made to produce fish which can grow faster, efficient in feed conversion in to body mass, resistance to diseases, and tolerant to various biotic and abiotic stresses. Number of workers have developed genetically improved tilapia for commercial aquaculture through selective breeding technique [8-11]. However, there is a restriction to the exploitation of Tilapia in developing countries due to the limited growth rate and excessive reproduction which results in fish that are small and variable in size. Transgenic techniques offer the means of producing the traits with targeted desired properties [9, 11]. However, ethical issues are yet to be addressed as far as transgenic fish is concerned.

In recent years, aquaculture and marine biotechnology is gaining lot of importance not only from the food security point of view but also due to its substantial contribution to our economy through the development of biotechnology industry. Billions of people all over the world are dependent on aquatic and marine biodiversity for their livelihood, day to day income generation, and employment opportunities. Biotechnology related to aquaculture and marine sciences can address number of future challenges particularly in the area of discovery of new products and processes of economic importance through rich biodiversity that is available in our seas and oceans in particular. At present continuous efforts are being made to exploit these resources on sustainable basis for their effective and judicious use as health food, in the field of pharmaceutical and nutraceutical industries, and in production of medicines and biomaterials. Recent research indicates that the marine biotechnology is now being heavily used to find out the alternate source of energies in the form of biofuels from the micro-organisms (micro-algae). The potential for the growth of marine biotechnology is, therefore, even more relevant now than it was in the past and a sound strategy for its development in India is being addressed to allow this potential to be realized and harnessed. Thus, Marine Biotechnology is capable of making inroads and important contribution towards meeting impending challenges in food production, energy, environment and human health sector [12].

In marine biotechnology, the domain areas of research that have been identified are disease diagnostics, vaccine development and immune-stimulant research along with the discovery of biomolecules that have anti-bacterial, anti-inflammatory, and anti-cancer properties. The research carried out so far in the development of suitable fish and shellfish cell lines and tissue culture systems will create the much needed support to enhance diagnosis of aquatic animal viruses and could lead to large scale production of vaccines. The gene mapping and development of molecular diagnostic methods of some shrimp viruses have been successfully carried out to address issues of biosecurity and manage disease problems by preventing their spread in aquaculture farms [12]. This has also facilitated the development of novel sensitive and specific DNA and antibody based diagnostics for the various fish, prawn and shrimp diseases. Recombinant protein vaccines developed for warm water fish pathogens such as *Aeromonas hydrophila, Edwardsiella tarda,* and *Vibrio anguillarum* have shown enhanced immune response and a high degree of protection against disease in laboratory trials and these products are ready for field trials. Viral vaccine for the dreaded nodavirus disease of Seabass, has shown promise in managing the associated mortalities in hatcheries. Since biotechnological approaches in aquaculture is expected to provide food security by enhancing production and reducing losses due to diseases, considerable research has been done into improving production by basic studies on breeding technologies, species diversification, developing genetically resistant stocks, transgenic approach to develop resistant and fast growing varieties, and development of appropriate feeds and feeding regimes including larval feeds [12]. Research is being carried out with regard to the utilization of waste materials such as chitinous shell of crustacean and collagen of fish for production of novel biomaterials with diverse applications [13, 14]. Research on marine derived biomaterials and molecules such as microbial enzymes and bio products that find application to human and animal health has also been prioritized through various funding agencies. Successful isolation of deep-sea fungi and other extremophiles, the cloning and expression of novel enzymes from them has resulted in promising technologies. To promote aquaculture of marine species, we need powerful technologies for accelerating maturation and growth process in fish and shellfish in captive condition. Eyestalk ablation technique for breeding and spawning of shrimp and prawns need to be replaced with an alternate technology based on molecular biology. This powerful technique may pave the way for regular breeding and spawning of commercial shrimps in captive condition and our requirement of enormous amount of seed can be fulfilled.

## Gene editing technology

Gene editing technology is gaining lot of importance in recent years to improve the quality of traits particularly in the agricultural commodities, animal and fisheries sector, etc. Researchers have focused on the application of three main types of gene editing techniques viz., ZFNs, TALENs, and the CRISPR/Cas9 RNA-guided endonuclease system. Each of these technologies utilizes restriction enzymes to introduce a DNA double stranded break at a targeted location with the guide of homologous binding proteins or RNA. Such targeting location and altering the defective portion of the gene structure is viewed as a significant advancement in genetic engineering when compared to current gene therapy methods that lack such specificity. In recent years, researchers have conducted several studies to evaluate gene editing tool using various experimental animals to treat multiple disorders related genetic defects including *in vivo* experiments in mammals and even early phase human trials [15-17].

CRISPR like concept was first discovered by the Japanese scientist while working on the DNA of bacteria in 1987 [18]. While working on protein-encoding gene in *E.coli*, they found a pattern of short, repeating, palindromic DNA sequences separated by short, non-repeating, "spacer" DNA sequences in the whole genome structure. Later, they reported that these repeats were present in many bacteria and other single-celled organisms. Jinek et al. (2012) while studying the RNA guided DNA endonucleases in adaptive bacterial immunity introduced the term 'CRISPR' to describe the pattern of DNA configuration in the bacterial genome. Further, while studying the CRISPR in details they discovered that the repeating DNA patterns, along with a family of "Cas" (CRISPR-associated) proteins and specialized RNA molecules, play a significant role in bacterial immune systems [19].

Concept of CRISPR was developed earlier by researchers while studying the bacterial immune system and adoption of defence mechanism in response to invading DNA molecule in the form of virus. Upon analysis of the bacterial immunity, it was observed that in response to invading DNA of the virus, CRISPR-Cas can copy the segments of foreign DNA molecule and incorporate the same in to their genome as "spacers" between the short DNA repeats in the form of CRISPR. These copied DNA molecules in the spacers actually protects the bacteria by strengthening their immune system by providing RNA molecule in the template form and identify and target quickly the same DNA molecule in the event of future viral infection. If these template RNA molecules identifies the incoming foreign DNA, then these template RNAs actually guide the CRISPR complex to that sequence. Further, the Cas protein that is present in the bacteria has the unique property of cutting DNA molecule and disabling the foreign invading DNA. From these important findings of the bacterial immune system reported by number of earlier workers [18], Doudna and Charpentier (2014) developed a technique of gene editing [20]. Later they named this technique as CRISPR-Cas technology [17, 19]. This technology involved CRISPR, a Cas protein called Cas9, and hybrid RNA that could be programmed to identify, cut, and even replace any gene sequence. The main advantage of the CRISPR technology over the other two gene editing technologies namely, ZFNs and TALENS is that the technology is cost effective and user-friendly. In ZFNs and TALENS technologies, there is a need of custom proteins for each targeted DNA molecule. As far as CRISPR-Cas9 technology is concerned, there are several reports regarding its successful usage in the embryos of mice, frogs, and monkeys, human stem cells, and immune cells. There are also published reports regarding exploration of different applications of CRISPR-Cas9 in genetically modifying agricultural crops, eradicating of viruses and screening of cancer genes. Doudna and Charpentier (2014) while working on genome engineering with CRISPR-Cas9 mentioned that there are number of techniques available to reduce the number of mutations at unintended sites in the animal system [20]. Now with advent of genome engineering technologies like CRISPR-Cas9, it is possible to intervene in the human genome function very precisely by editing undesired portion of DNA sequence. Here, the Cas9 can be guided to the targeted locations within the genome complex by a short RNA molecule. In recent times, several researchers have described that with this CRISPR-Cas9 technology, one can edit the DNA sequences within the endogenous genome and change the functional output virtually in any organism [2, 17].

Makarova et al. (2011) while working on the evolution and classification of the CRISPR-Cas systems reported three types of CRISPR mechanisms i.e. type I, type II and type III, each of which is characterized by signature proteins (Cas3, Cas9 or Cas10) [21]. Further it is mentioned that out of three types of CRISPR mechanisms, type II is the most studied one. Jinek et al. (2012, 2013) and Doudna and Charpentier (2014) while working on dual-RNA–guided DNA endonuclease in adaptive bacterial immunity mentioned the detailed mechanism of type-II CRISPR. In this mechanism they found out that when foreign DNA from viruses or plasmids invades in to the bacteria, this DNA molecule is immediately broken in to small fragments and get incorporated in to the targeted locus as short repeats. These loci are later transcribed and then the transcripts are processed to generate small RNAs (crRNA) which are used to guide effector endonucleases that target invading DNA based on sequence complementarity. To achieve site specific DNA recognition, Cas9 must be complexed with both a crRNA and a separate transactivating crRNA that is partially complimentary to crRNA [17, 19, 20].

In fisheries sector, in order to promote aquaculture/mariculture activities, industries related to this field are facing number of challenges as mentioned above, particularly in area of seed production as per the requirement, control of health and hygiene, production of quality traits with phenotypically improved varieties, and strengthening immune system. Several efforts are being made to develop techniques based on biotechnological knowledge or molecular genomics to meet these challenges and substantial achievements have also been made. However, with the advent of now recently developed gene editing technique, we may have to evaluate the possibilities of applications of this technology in addressing at least some of the important issues in the above mentioned areas. In shrimps and prawns, there are supressing/inhibiting factors in the eyestalk neuroendocrine complex which always prevents breeding and spawning in captive condition. Even the growth process is also hampered by these inhibiting factors. The immune system of these animals has been reported to be fragile and therefore, the chances of occurrences of viral and bacterial diseases are very common. Heavy losses have been reported by the shrimp or aquaculture industries due to the outbreak of diseases. Gene structure of gonad inhibiting hormone (GIH) and moult inhibiting hormone (MIH) in case of certain marine shrimps has already been investigated [22-24]. This structure of the gene can be altered/edited by using CRISPR-Cas technology to eliminate the negative impact of these hormones on reproduction and growth processes. This may pave the way to develop an alternative and powerful technique in place of eyestalk ablation with similar impact. Efforts have been made to silence/knockout this gene using the technique of RNA interference by some workers but the success achieved is not as per the expectations and further research is needed in this area [25, 26]. Treerattrakool et al. (2011) while working on *P. monodon* used the technique of RNA interference for inducement of maturation both in wild and captive shrimp and reported that shrimps injected with anti-GIH double stranded (ds) RNA showed advanced maturation [25]. Das et al. (2015) reported silencing of gonad inhibiting hormone gene in the eyestalk neuroendocrine complex of the tiger shrimp *P. monodon* by using the technique of RNA interference. They observed 3–5 fold enhanced expression of the androgenic gland hormone (AGH) transcript in males, but there was no effect on vitellogenin expression in females. In the destalked animals, however, positive effect on the maturation indicator transcripts was seen in both the sexes, and surpassed the efficiency of the silencing treatment [26].

The rapid advances being made in gene editing using the CRISPR-Cas9 system have led to increasing interest in the application of this technology in zebra fish embryos for gene knockout. Blasky et al. demonstrated that by using CRISPR-Cas9 in the embryos of zebra fish through microinjection, it is possible to knockout the functional protein gene by gene editing efficiency. To determine the efficacy of the Edit-R CRISPR-Cas9 in zebra fish, approximately 100 single-cell stage embryos were microinjected with Cas9 mRNA and synthetic cr-RNA, tracr-RNA, and approximately 50 control embryos were injected with Cas9 mRNA only. High survival rate of embryos after injection was observed (above 95%). Detecting functional knockout of Green Fluorescent Protein (GFP) was performed by imaging the zebra fish microinjected with Cas9 mRNA and crRNA, tracrRNA and comparing them to the embryos that were not injected. Following microinjection of CRISPR-Cas9 components targeting GFP, a loss or decrease in GFP fluorescence is observed in the zebra fish embryo, confirming successful gene knockout [27].

CRISPR-Cas technology can also be used to control the viral and bacterial diseases particularly in shrimps and prawns. The mechanism of CRISPR-Cas in shrimps/prawns may also work just like that of bacteria when they are invaded by virus DNA. For example, when WSSV invades shrimps, CRISPR-Cas can copy and incorporate segments of the WSSV DNA into their genome as "spacers" between the short DNA repeats in CRISPR. These spacers enhance the shrimp's immune response by providing a template for RNA molecules to quickly identify and target the same DNA sequence in the event of future viral infections. If the RNA molecules recognize an incoming sequence of foreign DNA, they guide the CRISPR complex to that sequence. There, the shrimp's Cas proteins, which are specialized for cutting DNA, splice and disable the invading gene. This may result in protecting the shrimp from infectious diseases. Therefore, a modern genome editing CRISPR- Cas9 technology can prove as a powerful tool in order to prevent the infection of viral and bacterial pathogens. CRISPR has potential of altering germ line of humans, animals, and other organisms. This approach can be achieved with the help of supporting tools like bioinformatics, primer design, and microinjection to identify and insert the CRISPR-Cas9 into the targeted genome.

More recently, the CRISPR-Cas9 system has greatly expanded the ability to *knock-out* genes in different animal models, including zebrafish [28, 29]. However, time and costs required for the screening of a huge number of animals are still a bottleneck. In fish, breeding and spawning in captive condition at our desired time is still not that easy. In advancing maturation by using hormonal techniques, limited success has been achieved. In such situations, is it possible to use CRIPSR–Cas technique to edit genes responsible to produce gonad stimulating hormones at desired time and maintain its hormonal titre in the body on continuous basis so that maturation process will be faster is a matter of investigation. Even the possibility to eliminate the *knock-out* of the undesired genes not favoring gonad development by using the CRISPR-Cas technique has to be investigated. Fish and shellfishes are very sensitive animals and are susceptible for infectious diseases. The common infectious vectors are

viruses, bacteria, fungi, and parasites. In fish farming systems occurrence of such infectious diseases is common affair which generally affects the growth and development of aquatic species. However, if the infections are of severe nature or in the event of outbreak of disease, there can be huge mortality and the whole farming operations may collapse. Therefore, controlling of diseases is one of the most vital tasks in fish culture system. Several methods are being developed and used including the fish vaccines to control the various diseases. But still whenever outbreak of disease occurs, heavy losses have been reported by the aquaculture industry. Therefore, we need to have powerful tools like gene editing using CRISPR-Cas technology to control the diseases particularly those which are caused by viral and bacterial infections. Recently, Brown et al. (2016) while working on multiplexed targeted genome engineering technology using nuclease assisted vector integration system reported that with the help of this technology one can knock out selected or targeted gene of the vector thereby minimizing the cost and time frame needed for gene editing. It has also been mentioned that this system is capable of remodeling native mammalian genome through DNA integration, up to 50kb, facilitating quick generation and screening of multigene knockouts from a single transfection. Furthermore, they have mentioned that this nuclease-assisted vector integration is a very strong tool for gene editing that will facilitate diverse application in gene therapy and synthetic biology [30].

In CRISPR system, gene knock out is carried out by introducing DNA double strand breaks (DSB) at the target locus and when these are repaired by error- prone DNA repair pathways, it causes inactivating mutations. It is reported that this system is laborious and costly for identification and isolation of isogenic cell lines which remains challenging in genetic engineering. Whereas in the case of multiplexed targeted genome engineering tool, there is mechanism of co-delivering engineered nucleases with donor vector containing expression cassettes that confer antibiotic resistance for rapid clonal screening. These donor vectors consists of two DNA sequences homologous to the region of DNA upstream and downstream of the intended DNA double strand break, flanking the heterogeneous DNA that will be incorporated into the genome following repair of DSB. Here, the donor vector stimulates DNA repair through homologous recombination pathway [31]. This method has been used successfully for multiple applications [32] including gene knock out, delivery of therapeutic genes [15, 33] or for tagging endogenous proteins [34-36].

## Conclusion

The Biotechnological challenges as far as aquaculture industry is concerned are many. The growth of the industry is not progressing as expected due to the lack of powerful scientific tools to control many factors at the farm level while culturing the commercial species in the aquatic environment. Heavy losses have been reported every year due to mass mortality, rejecting the consignment of aquaculture species attributed to lack of quality standards, biotic and abiotic stresses impact on aquaculture species, and no standard methods/protocols for disease control and pollution impact. Several attempts have been made using the knowledge of biotechnology to solve some of the problems of the aquaculture industry. However, at this juncture we need very powerful technologies to mitigate some of the severe issues that the aquaculture industry is facing. With the advent of the discovery of CRISPR Cas technology, there is a possibility that those biological issues related genetic disorders or otherwise can be solved safely without much altering the genetic structure of the aquaculture species.

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## Conflict of Interest

Authors declare no conflict of interest.

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