BACTERIAL STRAIN IMPROVEMENT FOR BIOMOLECULES THROUGH CHEMICAL AND PHYSICAL MUTAGENS

THESIS

By

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CERTIFICATE – I

This is to certify that the thesis entitled **"Bacterial strain improvement for biomolecules through chemical and physical mutagens"** submitted in partial fulfillment of the requirements for the award of the degree of **Master of Science** in the discipline of **Agricultural Biotechnology** of CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur is a bonafide research work carried out by **Akriti Thakur** daughter of Sh**.** Arun Kumar under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

Place: Palampur
Dated: March 2021

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CERTIFICATE- II

This is to certify that the thesis entitled **"Bacterial strain improvement for biomolecules through chemical and physical mutagens"** submitted by **Akriti Thakur (A-2018-30-001)** daughter of Sh**.** Arun Kumar to the CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur in partial fulfillment of the requirements for the degree of **Master of Science** in the discipline of **Agricultural Biotechnology** has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

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(Akriti Thakur)

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LIST OF ABBREVIATIONS USED

Abbreviation	Meaning	Abbreviation	Meaning
%	Percent	mM	Millimolar
µl	Microlitre	M	Molar
µM	Micromolar	Min.	Minute
µg	Microgram	MCT	Micro centrifuge tubes
A	Adenosine	MMS	Methyl methane sulphonate
°C	Degree Celsius	Nm	Nano metre
Conc.	Concentration	NB	Nutrient Broth
C	Cytosine	NaCl	Sodium Chloride
Sp.	Species	NMU	Nitrosomethyl urea
Viz.	Videlicet (namely)	NTG	N-methyl-N'-nitro-N-nitrosoguanidine
C	Cytosine	OD	Optical Density
T	Thymine	PHA	Polyhydroxyalkanoate
G	Guanine	PHB	Polyhydroxybutyrate
DNA	Deoxy Ribonucleic acid	PLA	Poly (Lactic Acid)
EMS	Ethyl methane sulphonate	PCA	Deoxy thymidine triphosphate
e.g.	Exempli gratia (for example)	PBS	Poly (butylenes succinate)
Etc.	Et cetera	PHV	Polyhydroxyvalerate
et al.	and others.	pH	Potential of hydrogen
Fig.	Figure	PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
g	g force (Relative Centrifugal Force)	Rpm	Revolutions per minute
H (h)	Hours	RNA	Ribonucleic acid
i.e.	Id est (that is)	Temp.	Temperature
Jm^{-2}	Joules per square metre	NA	Nutrient Agar
ml	Milliliter	UV	Ultra Violet
mg	milligram		

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ABSTRACT

Microorganisms are crucial life form present on earth, inhabiting all climatic zones including high altitude Himalayan niches. Microbes can thrive in harsh environmental conditions due to their ability to produce biomolecules such as enzymes and metabolites that perform specialised biological functions. Biomolecules produced by microbes are in minute quantities. Therefore, there is a need to increase the production for large scale applications through strain improvement. Hence, the current study was focussed on the strain improvement of unique bacterium *Iodobacter sp.* PCH194 through the application of chemical mutagens MMS, EMS, and NMU and physical mutagen in the form of UV radiation. The isolate PCH194 co-produces PHA and violacein, which has wide industrial applications. Through systematic applications of mutagens on wild-type PCH194, mutants with desired features were obtained and designated as IN1, IN2, IN3, IN4, and IN5. Their growth kinetics at alleviated temperature were observed. It was found that their growth temperature increased from 20 to 25°C, and slow growth was also observed at 28°C. However, the application of thermo protectants glycine betaine and glutamate could not significantly enhance the growth at 28°C. There was a marked increase in growth, PHA and violacein production of the mutants at 20°C. The PHA production was 1.24 mg/ml for IN5 and violacein production was 1.63 mg/ml for IN2, whereas wild strain produced 0.42 mg/ml PHA and 0.20 mg/ml violacein, respectively. In conclusion, the present study successfully increased the growth temperature of *Iodobacter sp.* PCH194 from 20°C to 25°C and also enhanced the production of PHA and violacein. Hence, generated mutants can further be used for process optimisation and scale-up studies.

Keywords: MMS, EMS, NMU, PHA, violacein, physical mutagen, chemical mutagen, thermoprotectants, *Iodobacter sp.*, bacteria, industrial production

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1. INTRODUCTION

Microorganisms are important life form present on the earth. They inhabited all climatic zones, including extreme environmental niches such as hot springs, hydrothermal vents, acidic, alkaline soils and low temperature such as Arctic, Antarctic and high-altitude mountains regions. Himalaya is the largest and highest mountain range in the world and poses life form with extreme environment stresses. The Himalayan region provides a tremendous opportunity to understand the adaptability of microorganisms to an extreme environment that can be utilized for industrial applications (Thakur et al. 2018; Kumar et al. 2018; 2020; 2021). Microbes can thrive in such harsh environmental conditions due to their ability to produce unique enzymes and metabolites that perform specialized biological functions. Therefore, the study of microbial diversity of Himalaya is important to identify and characterize microbes for efficient novel genes/enzymes of industrial importance (Amico et al. 2006; Kumar et al. 2014).

Most of the bacterial species produce biomolecules that are unique and are of commercial importance. The wild-type bacteria produce biomolecules to their physiological needs therefore, yields are usually low to meet industrial and commercial demands. Therefore, strain improvement is required to enhance the production of the biomolecules. Different approaches are available to improve the wild strain in terms of specific characteristics like yield, efficiency, stability, and specificity (Bapiraju et al. 2004; Upendra and Khandelwal 2016; Lim et al. 2018; Kamalambigeswari et al. 2018; Mehmood et al. 2019; Sayed et al. 2019). The basic mechanism of strain improvement is to alter the genomic DNA sequence, which can be achieved by random mutations, site specific mutations, genetic engineering, and genetic recombination. Many times, a trait is controlled by multigene factors. Hence, *in vitro* expression or site directed mutagenesis is not a profitable business. The classical genetic approach to improve the desirable trait(s) and make the organism more effective is by subjecting them to random mutations using mutagenic agents and

screening the survivor's colonies for a desired trait(s) (Parekh 2009; Kumar et al. 2015).

Mutation brings about an alteration in the sequence of genes controlling the specific characters. A wild bacterial strain is subjected to physical (UV radiation) and chemical mutagens (ethyl methane sulfonate, methyl methane sulfonate etc.) that bring about alterations at the DNA level in a microorganism (Snustad and Simmons 2012). UV rays give a high proportion of pyrimidine dimers, produce hydroxylated bases and cross linking of DNA strands. In contrast, chemical mutagens such as EMS and NMU are alkylating agents that donate alkyl group to the DNA bases resulting in altered base-pairing. In the past, mutation has been extensively used for enhanced production of biomolecules polyhydroxyalkanoates, penicillin, laccase, lactic acid etc.(Weenink et al. 2006; Adrio and Demain 2006; Hungund and Gupta 2010; Weber et al. 2012; Obruca et al. 2013; Derkx et al. 2014; Kumar et al. 2015; Fiedurek et al. 2017; Lim et al. 2018; Sayed et al. 2019).

A number of bacteria from the Himalayan region have been isolated, characterized and explored to produce bioplastic and lignocellulose degrading enzymes of necessary groups at CSIR IHBT (Thakur et al. 2018; Kumar et al. 2019; Kumar et al. 2018; 2020; 2021;). The present study is focused on a unique bacterium *Iodobacter* sp. PCH194 isolated from high altitude region of western Himalaya in a laboratory at CSIR IHBT, Palampur. The bacterium can produce bioplastic and a violate colored pigment violacein at 20°C (Kumar et al. 2021). Despite of good co-production of both the molecules in a single bioprocess, the bacterium has a growth optimum at 20°C. Therefore, it requires lower temperature to maintain 20°C during fermentation that may add additional cost to the developed process. In light of the above, chemical and physical mutagens are used in this study to create mutants that can grow above ambient temperature suitable for industrial-scale fermentation. Hence, the study has envisaged the following objectives:

1. Generation of mutants using physical and chemical mutagenesis.
2. Evaluation of mutants for their biological activity.

2. REVIEW OF LITERATURE

Microorganisms can thrive in almost any extreme habitat *viz.* cold, heat, radiation, pressure, salt, acidity and darkness via the usage of a variety of energy sources (Satyanarayana et al. 2013; Mehta and Satyanarayana 2013; Joshi et al. 2017; Kumar et al. 2018). Altitude is the crucial factor that has confounded effects on physiochemical properties and biodiversity of the niches. High altitude regions usually have low temperature, variable precipitation, and decreased atmospheric pressure .Such environment factor lead to variation in soil fertility characteristics like, organic carbon, pH, total nitrogen, phosphorus, and micronutrients thus creating a major impact on biodiversity (Turner et al. 2013; He et al. 2016). The majority of the biosphere on earth constitutes cold environments that have been colonized by cold-adapted microorganisms. These microorganisms maintain their metabolic activity even at subzero temperature and can survive and thrive in the extreme environments (Nunn et al. 2015; Koh et al. 2016). There are reports of bacterial diversity in soil from Rohtang Pass's altitudes (Yadav et al. 2015) and Pangi-Chamba (Kumar et al. 2018; Thakur et al. 2018) situated in North-western Indian Himalaya. Proteobacteria was found the major phyla found followed by Firmicutes, Actinobacteria and Bacteroidetes (Ganwar et al. 2009; Kumar et al. 2018). These microorganisms exhibited remarkable plant growth promontory properties, antifreeze proteins, membrane fluidity, cold, and heat-shock responses (Feller and Gerdy 2003; Chintalapati et al. 2004; Amico et al. 2006). Similarly, microbial diversity from Sikkim Himalaya revealed the presence of bacteria and fungi, which decreased along the increasing altitude (Rai and Kumar 2015).

2.1 Psychrophiles / Psychrotrophs

Psychrophiles are microorganisms that flourish at low temperature, having an optimal growth temperature between 15 to 20°C and minimal growth can be 0°C or lower (Feller and Gerday 2003). Whereas, psychrotrophs have similar properties as psychrophiles except it can grow at a slightly higher temperature range of 20 to 25°C (Moyer and Morita 2007). They have evolved by adjusting to these conditions

through various synergistic adaptations, including presence of various metabolic pathways for endurance and habitation in permafrost conditions (Kawahara 2017). A few specialized features such as cell envelope adapted to the external factors, cryoprotectants synthesized within the cells, new chaperones and novel metabolic capabilities are developed over the ontogeny of the microorganisms (Amico et al. 2006; Joshi et al. 2017). In addition, these organisms have been reported to conserve energy for long term survival via accumulation and metabolism of reserve compounds (Medigue et al. 2005; Piette et al. 2011; Tribelli et al. 2015; Tribelli and Lopez 2018).

The Himalayan region is replete with psychrophillic/psychrotrophic microbes that produce biomolecules of potential interest to human kind (Swarnkar et al. 2014; Yadav et al. 2016). Biomolecules exhibiting antifreeze, antibiotic, extracellular hydrolytic properties, and bioactive compounds with potential biotechnological applications in pharmaceuticals, medicine, food, and feed industry are the forte of these organisms (Gerday et al. 2000; Yadav 2015; Singh et al. 2016). These cold adapted microbes are also documented to produce biofuels, and biodiesel having possibility to implement in future energy systems (Kawahara 2017).

2.2 Biomolecules from microbes

The medley of microorganisms in the biosphere is responsible for global primary energy and element cycling (Beloqui et al. 2008). In terms of total biomass and cell numbers, they represent the most abundant source for biological activity (McHardy and Rigoutsos 2007; Ward et al. 2008). The biological interactions in nature are mostly associated with the discovery biomolecules from microbes (Shi et al. 2007). The complexities and interactions among the microbes and their environment, constitutes the core in understanding and finding effective solutions for industrial needs (Woodley 2006; Schmeisser et al. 2007; Beloqui et al. 2008; Venil et al. 2013).

Since antiquity, microbes have been utilized by humans for a variety of purposes. Reports from 6000 BC suggest that Indus valley, Babylonians, and Sumerians people had used yeast to produce alcoholic beverages from Barley (Singh et al. 2016). The discovery of various novel antibiotics in the 1970s from microbes significantly increased academia and industry's attention towards microbial biotechnology (Singh and Pelaex 2008). The beneficial microbes and their biomolecules of interest were

used as potential constituents for the wide-range of natural product-based preparations and formulations (Adrio and Demain 2006; 2008; 2014). Currently, microbes are used as a source for various drugs, vitamins, biofuels, enzymes, bulk organic compounds, polymers, amino acids and antibiotics, with tremendous application in biopharma, agriculture, food processing and preventing human diseases (Gupta et al. 2011; Li et al. 2012; Choi et al. 2015; Saxena 2015; Sharma et al. 2019).

Out of all known natural products, it is estimated that bacteria are responsible for the majority of the produce of microbes. Of these, nearly 3/4thof the products are produced by *Actinobacteria,* consisting of the most prolific genus *Streptomyces* (Berdy et al. 2005). A number of studies established that 75% of antibiotics are produced by *Streptomyces* and *Actinomycetes* (Omura 1992; Miyadoh 1993; Zedan 1993; Lazzarini et al. 2000; Berdy 2005). The commercial production of amino acids, vitamins, ethanol and antibiotics are carried out either by fermentation or genetic engineering (Moniruzzaman and Ingram 1998; Demain et al. 2005; Survase et al. 2006; Van et al. 2012; Sun et al. 2015). A large proportion of pharmaceutical agents *viz.* immunosuppressants, enzyme inhibitors, anti-cancer /antitumor agents, and drugs are produced by bacteria (Hopwood et al. 2000; Rodgers et al. 2012).

For commercial-scale production, penicillin from *Penicillium* sp., alginates from *Pseudomonas* sp, *Azotobacter* sp; cellulase from *E. coli*, *Bacillus* sp. and hyaluronic acid from *Bacillus* sp., *Streptococcus* sp. and *Staphylococcus* sp., are being produced (Widner et al. 2005; Weber et al. 2012; Hay et al. 2013; Sadhu and Maiti 2013; Saranraj and Naidu 2013). However, the production and wide scale application of many other important biopolymers is still at infancy. Concerns over the environment pollution have opened up new frontiers on development of biodegradable biopolymers. The major world powers are scouring through resources to find an alternative to plastic that is the single most hazardous substance plaguing our planet. Biopolymers like polyhydroxyalkanoates (PHAs), that are deemed to be quite valuable for humanity and the environment; due to their plastic like properties. However, for the commercial feasibility researchers are still struggling for cost effective production at the industrial level. The majority of biomolecules, while having a great significance in different bio-sectors, are still in research and development or early commercialization phase in terms of their production process.

2.3 Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are biologically synthesized polymers of hydroxyalkanoic acids through repeated ester bonds among the monomer units as shown in Fig. 1 (Sehgal and Gupta 2020). PHA was first discovered by Lemoigne in the *Bacillus* sp. (Lemoigne 1926). However, the production of PHA was explored truly in the 1960's on a commercial scale (Philip et al. 2007). PHA can be homopolymers, heteropolymers or co-polymers based on the number of carbon atoms in the chain and the type of monomer (Perez et al. 2019). The PHA are classified into two groups *viz.* short chain (scl- PHA) consisting of 3-5 carbon atoms in their monomer and medium chain (mcl- PHA) having 6-14 carbon atoms in their monomer (Taguchi and Doi 2004). The most common PHA monomers are hydroxybutyrate and hydroxyvalerate (Bengtsson et al. 2008). PHAs are relatively resistant to hydrolytic degradation and UV radiation but are poorly resistant to acids (Bugnicourt et al. 2014).

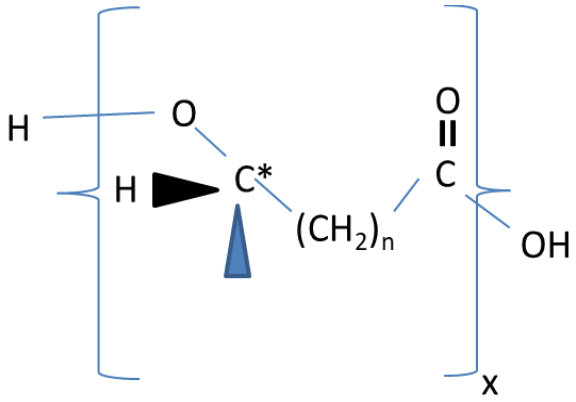

Fig. 2.1: Chemical structure of PHA.

2.3.1 PHA biosynthesis

Microbes produce PHA mainly via three metabolic pathways, involving either acetyl-CoA or acyl-CoA as intermediate and finally polymerization by PHA synthases (Fig. 2) (Philip et al. 2007). Pathway I utilize sugars as carbon sources and consist of three distinct enzymes PHA synthase, β-ketothiolase and acetoacetyl-CoA reductase

(Senior and Dawes 1973; Tsuge et al. 2003). Pathway II utilizes fatty acids as carbon sources and occurs in the presence of two main enzymes PHA synthases and enoyl-CoA hydratase (Sehgal and Gupta 2020). Whereas, pathway III is of greatest interest as it utilizes simple and inexpensive sugars (glucose, fructose and sucrose) as carbon source and occurs in the presence of enzyme acyl-ACP-CoA transacylase (encoded by phaG) (Philip et al. 2007).

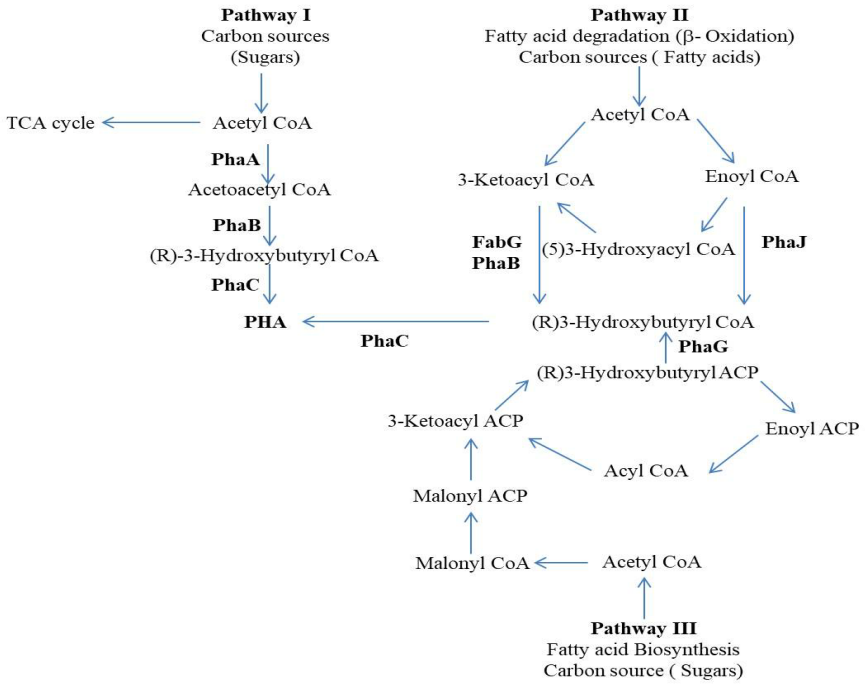

Fig. 2.2: PHA metabolic biosynthesis pathways (Adopted from Philip et al. 2007) (PhaA: 3-ketothiolase; PhaB: (R)−3-ketoacyl-CoA reductase; PhaC: PHA synthase or polymerase; PhaG: (R)−3-hydroxyacyl ACP:CoA transacylase; PhaJ: (R)-specific enoyl-CoA hydratase).

2.3.2 PHA biodegradation

The property that distinguishes PHA from traditional petroleum-based plastics is their ability to biodegrade (Anjum et al. 2016). Various bacteria and fungi can degrade PHA (Mergaert and Swings 1996; Methe et al. 2005; Lopez et al. 2009; Ting et al. 2010). PHA is biodegraded to carbon dioxide and water under aerobic conditions, while, under anaerobic conditions methane is produced instead of water (Shah et al. 2008). Copolymers such as the Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and polyhydroxyvalerate (PHV) have been found to be degraded more rapidly than PHB (Bugnicourt et al. 2014). It was observed that in different

ecosystems landfill leachate, sewage sludge compost, sewage sludge supernatant, forest soil, etc. The aliphatic polymer-degrading microbes, degrades biopolymers in the following order: PHB = PCL > PBS > PLA (Nishida and Tokiwa 1993).

2.3.3 Applications of PHA

The applications of PHA have increased exponentially along with time in drug delivery, medical implants, printing materials, nutritional supplements in addition to packaging materials (Bucci et al. 2007; Darani and Bucci 2015; Kalia et al. 2019; Singh et al. 2019). PHA has also been used as a type of biofuel (Chen 2009). PHA acts as a gas barrier which deems it suitable for its usage as a packaging material in the food and beverage industry (Fu et al. 2014; Prasad and Kochhar 2014; Albuquerque and Malafaia 2018). Recently, polymer films with desirable features such as high flexibility, nontoxicity, odorless, antioxidant and antimicrobial activities have been developed (Reis et al. 2016; 2017; Kiran et al. 2017). In the medical industry, PHA polymers are used in orthopedic as scaffolds and for engineering diverse mammalian tissues (Lobler et al. 2002; Qu et al. 2006; Chen 2010; Ching et al. 2016; Shishatskaya et al. 2016; Contreras et al. 2017; Dalal and Lal 2019). In the quest to make agriculture sustainable, PHA has been used as mulching material, plant growth promoter and nano-herbicide (Hassan et al. 2006; Vinet and Zhedanov 2010; Grillo et al. 2010; Lobo et al. 2011).

2.3.4 Challenges in commercializing PHAs

A major limiting factor for the commercial production of such biopolymers, especially PHA is their high production cost contributed majorly by the cost of feedstock such as carbon source and efficiency of carbon to PHA conversion rate (Valappil et al. 2008; Castilho et al. 2009; Singh et al. 2015; Kalia et al. 2019). In order to minimize cost, one strategy is to use a cheaper substrate. Minimizing the energy consumption during fermentation can also add to the cost reduction. Another unique approach is the expansion of the varieties of valuable products obtained from a single batch. Hence, a desirable aspect is the simultaneous production of two or more microbial products via the same process. This encapsulates a simplistic approach where there is a potential to reduce the cost whilst rendering a simple operation

(Sukan et al. 2015). Thus, for the commercial viable production of PHA, utilizing of low cost substrates, and generating mutants with high PHA-yields are most desirable.

2.4 Violacein

Violacein is purple-colored bis-indole pigment (Fig 2.3) produced by some bacteria of the genus *Chromobacterium, Iodobacter, Janthinobacterium, Alteromonas, Pseudoalteromonas, Duganella,* and *Collimonas* (Duran et al. 2016; Vishnu and Palaniswamy 2016; Kumar et al. 2021). Violacein exhibits maximum UV absorbance at 258, 372, and 575 nm, whereas the fluorescence emission spectrum shows an emission band at 675 nm at an excitation wavelength of 575 nm (Duran et al. 2007). Its melting point is above 290°C.

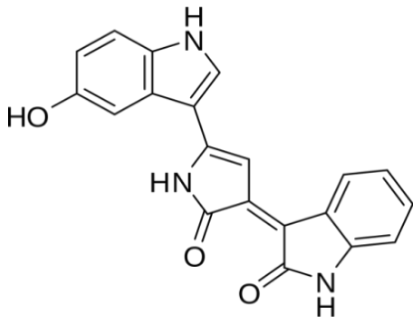

Fig. 2.3: Chemical structure of violacein.

2.4.1 Violacein Biosynthesis

Violacein biosynthesis pathway was first studied by Pemberton et al. (1991) and then was further investigated by Balibar et al. and Sanchez et al. in 2006. They concluded that violacein biosynthesis involves joint action of five enzymes (VioA, VioB, VioC, VioD and VioE) on the substrate L-tryptophan in the presence of oxygen (Fig. 2.4). The carbon and nitrogen atoms in violacein are derived from L-tryptophan, while one of the oxygen atoms comes from molecular oxygen (Balibar and Walsh 2006; Fang et al. 2015).

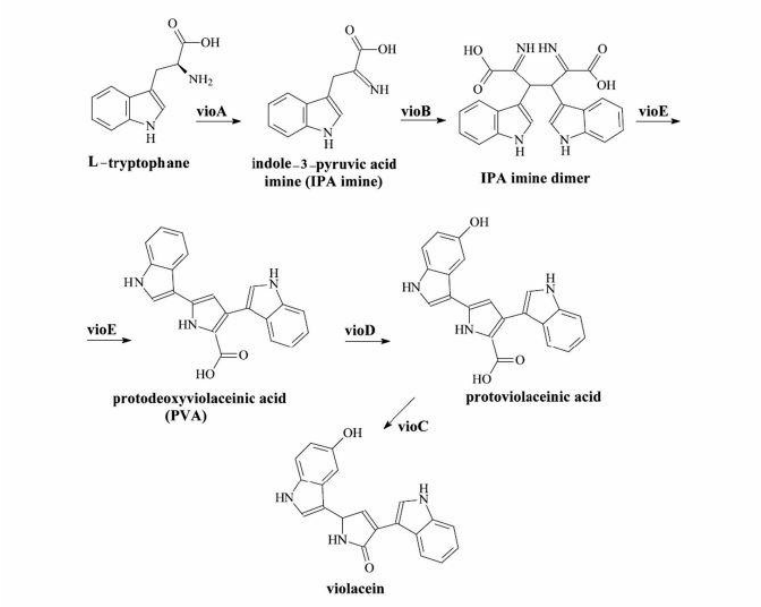

Fig. 2.4 Illustration of the violacein biosynthesis (Adopted from Hoshino 2011).

2.4.2 Biological activities of violacein

Violacein exhibits several biological activities with clinical importance that include antimicrobial, antioxidant, photoprotective and antitumor activities, leishmanicidal, trypanocidal, and immunomodulatory potential **(**Duran and Menck 2001; Duran et al. 2007; 2010; 2012; Soliev et al. 2011; Choi et al. 2015). It has an antimicrobial effect against gram positive bacteria and a weaker against gram negative bacteria (Cazoto et al. 2011; Kumar et al. 2021). The fungicidal activity was established by Shirata et al. (1997) and it was found effective against pathogenic fungi and yeast (Sasidharan et al. 2015). Violacein was also found to be effective against virus (HSV and poliovirus) and parasites (Duran et al. 2007). In addition, it was found to possess antioxidative (Rettori et al. 1998; Azevedo et al. 2000; Konzen et al. 2006; Duran et al. 2007) and antitumour properties and has been extensively studied on Myeloid Leukaemia and TF1 Leukaemia cells (Ferreira et al. 2004; Queiroz et al. 2012). In recent years, its toxicity towards eukaryotic cells was studied, which led to the understanding of its anti-helminthic property (Ballestriero et al. 2014). The findings from different researchers led to the understanding of the immunomodulatory potential of violacein (Antonisamy and Ignacimuthu 2010; Antonisamy et al. 2014; Verinaud et al. 2015). Hence, as a biomolecule, tremendous benefits that it imparts might be a game changer in future biomolecule applications.

2.4.3 Potential industrial applications of violacein

Violacein has many potential industrial applications in biotechnological and pharmaceutical fields. Violacein and its derivatives trans-hydroxyviolacein and deoxyviolacein are used in cosmetic industries in combination with lipophilic or hydrophilic substances (Duran et al. 2016). It is also being tested as a food colorant in yogurts and jellies. Violacein and its derivatives are used to color the fibers and silk fabric (Duran et al. 2012). The violacein-colored fabrics showed antimicrobial activity to phytopathogenic fungus (Shirata et al. 1997; 1998). The commercial applications of violacein can also include protecting human skin from sun light since it has antioxidant and antimicrobial activities (Suryawanshi et al. 2015). In the agricultural field, violacein derived from *C. violaceum*, *A. violacea* or *J. lividum* is mainly used for plant pathogens control (Baek et al. 2007; Duran et al. 2016). Natural pigments are being employed in the toy, food, and textile industries (Tan et al. 2011). Violacein has great potential in various domains of the life most of which have not yet been fully exploited. This can be attributed mainly to the low microbial production which in turn leads to higher cost. This problem can be solved through the application of strain improvement techniques in microorganisms.

2.5 Strain improvement

Microorganisms tend to produce metabolites of industrial value in low quantities. Therefore, industrially relevant microbes are subjected to various strain improvement techniques to enhance their synthetic capabilities. The manipulation of microbial strains and their improvement for the purpose of enhanced metabolic capacities for biotechnological applications constitutes strain improvement (Gonzalez et al. 2003). To improve the microbial strains, the gene sequence must be altered and/or manipulated. Thus, reprogramming and altering the DNA sequence to bypass the regulatory controls in a desired fashion constitutes the core of microbial strain improvement (Demain and Adrio 2008).

The development in strain improvement started in the 1940s when the production of penicillin became a necessity. These studies were based on the generation of mutants via application of physical and chemical mutagens. In the late 1980s and early 1990s, genes were mutated randomly through mutagens or error prone PCR, and

subsequently screened for improved functions. This approach gradually turned out to be a major step to routinely improve the functioning of biological macromolecules (Gonzalez et al. 2003; Cardayre 2005). The three main approaches that have been used to improve microbial strains are mutagenesis, recombination and recombinant DNA technology (Parekh et al. 2000). In recent years, with the advancements in manipulative genetic techniques, more targeted approaches are now used *viz.,* transposon mutagenesis, protoplast fusion, genetic engineering, etc. (Adrio and Demain 2006; Saxena 2015; Fiedurek et al. 2017).

In present times, the large-scale production of antibiotics, enzymes, and other biomolecules serve as a testimony towards strain improvement in shaping the pharmaceutical and fermentation industries (Demain and Davis 1998). The major goal of strain improvement is the volumetric production of natural products. Other criteria are purity of the product, tolerance to optimal conditions, etc. If this goal is not achieved at the genomic level, then optimization of its external environment can be done either by using thermo-protectants or osmo-protectant (Caldas et al. 1999; Holtmann et al. 2003). Hence, to proliferate the optimum level of growth in the microorganisms, strain improvement should be carried out in conjunction with the application of growth additives and stress protectants.

2.5.1 Mutagenesis

The mutation for strain improvement has been the preferred choice mainly due to its simplicity. Random mutagenesis requires no molecular or genetic tools or genomic information, except an effective mutagen and an accurate screen for the desired phenotype. However, this classical strain improvements approach is unpopular at the commercial level because of its time and labor-intensive nature (Demain and Adrio 2008).

2.5.2 Genetic engineering

Genetic engineering is the process involving recombinant DNA technology to alter the genetic makeup of an organism. This approach has been applied in industrial microbiological interests, as it can create multiple beneficial mutations, and allows specific control between beneficial and unwanted genes (Han and Parekh 2005). The first proof of genetic engineering for strain improvement came from the studies of

Thompson et al. (1982) on *Streptomyces* that used plasmid from *S. lividans* and *S. coelicolor* as cloning vehicles. This study discovered that genes conferring antibiotic resistance to strains are transferred to infective bacteria via plasmids. Since then, it has been used for production of enzymes, antibiotics, and other secondary metabolites (Chiang 2004; Adrio and Demain 2005; 2010; Pickens et al. 2011). Due to its precision and versatility, it would have been an ideal technology for enhancing microbial performance. However, it is difficult to know the target gene locations, and all the characters are not influenced by a single gene. Thus, to improve strains for industrial application, the natural strategy of physical and chemical mutagenesis is of significant interest (Derkx et al. 2014).

2.6 Mutagenesis

Modification of genes through spontaneous or induced mutation constitutes mutagenesis, which in turn causes sudden heritable change known as mutation. The strain harboring the mutation is called a mutant, and the agent causing the mutation is called a mutagen (Saunders and Saunders 1987). Therefore, a mutagen can be described as a chemical or physical agent that increases the rate of mutation beyond the spontaneous rate, and the three main mutagens used are physical, chemical, and biological mutagens. A detailed description of physical and chemical mutagens and their mode of action to alter the DNA are listed in the Table 1.

Table 2.1: Commonly used mutagens and their impact for strain improvement (Adopted from Parekh 2009).

Mutagen	Mutation induced	Impact on DNA	Relative effect
Physical			
Ionizing radiation			
X rays, Gamma rays	Single- or double-strand breakage of DNA	Deletions, structural changes	High
Short wavelengths			
UV rays	Pyrimidine dimerization and cross links in DNA	Transversion, deletion, frameshift, GC>AT transitions	Medium
Chemicals			
Base analogues			
5- Chlorouracil 5-Bromouracil	Faulty base pairing	AT->GC, GC->AT transition	Low
2-Aminopurine	Errors in DNA replications		Low
Deaminating agents			
Hydroxylamine	Deamination of cytosine	GC->AT transitions	Low
Nitrous acid	Deamination of A, C and G	Bidirectional translation, deletion, AT->GC, and/or GC->AT transition	Medium
Alkylating agents			
NTG	Methylation, high pH	GC->AT transitions	High
EMS	Alkylation of C and A	GC->AT transitions	High
NMU	Alkylation	AT->GC transitions	Low
Mustards, di-(2-chloroethyl) Sulphide	Alkylation of C and A	GC->AT transitions	High
Intercalating agents			
Ethidium bromide, acridine dyes	Intercalation between two base pairs	Frameshift, loss of plasmids, microdeletions	Low
Biological			
Phage, plasmid, DNA transposons	Base substitution, breakage	Deletion, duplication, insertion	High

2.6.1 Physical mutagens

Radiations are the high-energy levels of the electromagnetic spectrum that dislodge the electrons from the nuclear orbits of the atoms that they impact upon. Radiation was the first mutagenic agent known and its effects on genes were first reported in the 1920s. When passing through tissue it causes ionization leading change in DNA, membranes, lipids, and enzymes, etc. *viz.* formation of activated molecules i.e., free

radicals (Erixon and Ahnstrom 1977). The electromagnetic spectrum is divided into ionizing and non-ionizing radiation. Ionizing radiation constitutes X-rays, gamma rays, and cosmic rays while the non-ionizing radiation constitutes UV light (Mba et al. 2012).

Ionizing radiations

These are high energy rays that create positively charged ions or free radicals when colliding with atoms (Massey et al. 1953). Thus create a chain reaction which generates a series of ions as they pass through living tissues (Criswell et al. 2003). Some examples are X rays, protons, neutrons, alpha, beta, and gamma rays released by radioactive isotopes such as $Uranium_{238}$.

Non-ionizing radiations-

In contrast to ionizing radiations, UV rays having lower energy penetrate only the surface layer of the cells, hence is unable to cause ionization. UV rays transfer their energy to the outer orbitals raising the energy levels called excited state. The increase in reactivity of the atoms present in DNA results in mutagenicity (Demain and Adrio 2008; Oladosu et al. 2016). The maximum absorption of UV radiation by DNA is at 254 nm wavelength (Saxena 2015). The fact that maximum mutagenicity occurs at this wavelength also suggests that directly mediates the mutation process the absorption of UV by purines and pyrimidines which results in pyrimidine hydrates and pyrimidine dimers (Fig. 5) (Snustad and Simmons 2012). Specific mutations *viz.* cytosine (C) →thymine (T) and CC → TT occur. The former occurs at dipyrimidine sites and the latter occurs in a tandem manner which occurs rarely. These two types of mutation are also called UV signatures (Tessman and Kennedy 1991; Tessman et al. 1992). The mutability of UV radiation has been studied extensively due to its ubiquitous nature and ease of handling. Bridges et al. (1968) have postulated that application of UV causes DNA lesions with single-strand gaps or DNA break. Thus, UV has widespread usage in clinical microbiology labs and evolution engineering in biotechnology.

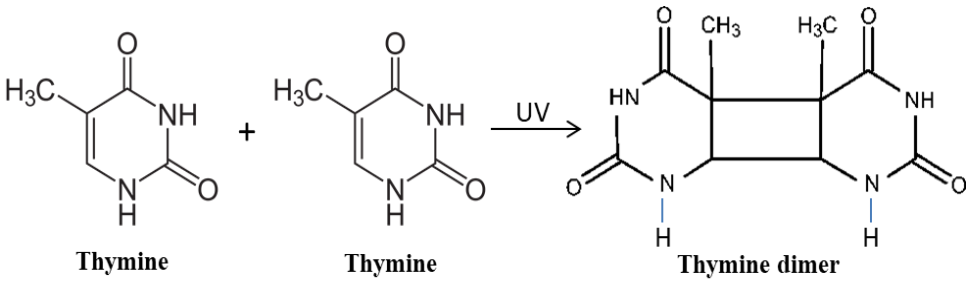

Fig. 2.5: Conversion of thymine into thymine dimer on exposure to UV.

2.6.2 Chemical mutagens

Chemical mutagens are the chemicals that induce changes in the DNA. Amongst, alkylating agents can mutate both the replicating and non-replicating DNA. The base analogues induced mutation only to replicating DNA and exhibit their effects during replication by increasing the frequency of mis-pairing (Saxena 2015; Oladosu 2016). Some of the most commonly used base analogues are 2-aminopurine and 5-bromouracil. On the other hand, a special type of mutagens, called acridine dyes intercalate into the DNA strand, increasing the probability of mistakes during the replication process (Anderso 1995).

Alkylating agents

These are chemicals that carry out mutation by donating alkyl groups to DNA bases. Some of the chemicals used as alkylating agents are ethyl methane sulfonate (EMS), methyl methane sulfonate (MMS) and nitrogen mustard (Singer and Kusmierek 1982). Alkylating agents are also classified as radiomimetic agents since their effects are similar to ionizing radiations (Drake and Baltz 1976; Kodym and Afza 2003). As chemical mutagens EMS, MMS and N-nitroso-N-methyurea (NMU) are known to be effective and efficient and are quite popular chemical mutagens. To their functional groups, chemical mutagens are mono, bi or polyfunctional alkylating agents (Kodym and Afza 2003). For many year MMS and EMS have been used as a DNA damaging agent for induction of mutagenesis and for recombination experiments. All types of mutations (transition, transversion, frameshift, chromosomal aberration) are exhibited by alkylating agents. They transfer the methyl or ethyl group to the bases which result

in altered base pairs e.g., EMS causes ethylation of bases in DNA at 7-N and 6-O positions which results in G:C → A:T transition. Some of them cross links DNA strands. Hence, as a whole, they are generating fewer specific mutagens (Oladosu et al. 2016).

2.7 Microbial strain improvement using mutagenesis

Physical and chemical mutagens have been used extensively to increase the biological activities of bacteria and other industrially significant microorganisms (Sauer 2001). Several studies have been carried out to improve the bacterial strains along with their associated properties and biological activities via the application of chemical and physical mutagens. They have been employed successfully for strain improvement particularly in the antibiotics and brewing industries e.g., production of antibiotics penicillin and cephalosporins (Elander 1967; Elander et al. 1976). Subjecting UV radiations to *Rhizopus* sp. BTS-24 enhanced its lipase production by 164% than its parent strain and 180% times higher than the wild strain (Bapiraju et al. 2004). Singh et al. (2016), improved the saprophytic capabilities of *Trichoderma* sp. through the use of chemical mutagen *viz.* NTG. Strain improvement of *Aspergillus niger* through its treatment with EMS has been carried out by Kamalambigeswari et al. (2018), indicating a 12% increase in enzyme production. Saini et al. (2020) concluded that treatment of MMS, EMS and NMU to *Saccharomyces cerevisiae* led to 46-fold, 39-fold and a 12-fold increase in mutation frequencies, respectively. Chauhan et al. (2020) developed a patent using a robust mutant, exhibiting enhanced xylanase activity through the application of EMS, wherein it was envisioned to be suitable for industrial applications. Several examples of effect of mutagen on the production of biomolecules are listed in the Table 2.

Table 2.2: Effect of mutagen on the production of biomolecules using bacterial system.

Organism	Mutagen used	Biomolecules	Improvement	Reference
Bacillus licheniformis	N-methyl-N-nitro-N-nitrosoguanidine	Lipopeptide biosurfactant	12 fold	Lin et al. 1998
Aspergillus sp.	UV, Colchicine EMS	Verbenol	15, 2 and 8-fold	Agrawal et al. 1999
Penicillium sp.	UV, Colchicine EMS	Verbenol	8, 1.5 and 2-fold	Agrawal et al. 1999
Penicillium janthinellum	EMS and UV	Cellulase	2 fold	Adsul et al. 2007
Gluconacetobacter xylinus	UV, EMS	Cellulose	30 and 98%	Hungund and Gupta 2010
Bacillus licheniformis	UV, N-methyl-N'-nitro-N-nitrosoguanidine	Polyhydroxybutyrate	3.18- fold	Sangkharak and Prasertsan 2013
Bacillus megaterium	UV	Polyhydroxybutyrate	2 fold	Girdhar et al. 2014
Gordonia terrae	EMS, MMS ENU	Nitrilase	2.5-fold	Kumar et al. 2015
Phellinus igniarius	He-Ne Laser UV	Endo polysaccharides	40.31 and 56.58 %	Zhang et al. 2016
Synechocystis sp. PCC 6714	UV	Polyhydroxybutyrate	2.5-fold	Kamravamanesha et al. 2018

2.7 Thermo-protection

Temperature affects membranes, RNA, DNA, ribosomes, protein, and enzyme activities of microbes incurring profound effects on their structural and physiological properties (Russell 2003). In order to negate this, many bacteria release some chemicals to maintain their growth and survival. Thermo-protectants elevate the temperature withstanding capacity of the bacteria by compaction of its molecular packaging. The most effective thermo-protectant used commonly for *E. coli* and *Bacillus* sp. are proline, glycine betaine, glutamate, and choline (Caldas et al. 1999; Holtmann and Bremer 2004). The growth of *Bacillus subtilis* JH642 was enhanced by applying glycine betaine and glutamate in low concentrations (Holtmann et al. 2004;

Holtmann and Bremer 2004). The thermal stability and specific activity of lipase from *Psychrobacter* sp. in the presence of betaine and trimethylamine-N-oxide enhanced the activity of lipase (Santi et al. 2012). Thermo-protectants, thus, are a remarkable option to maintain the viability of the microbes in hostile environment, i.e., proliferation of psychrophiles at room temperature which would pave way for their industrial applications.

Conclusions

Microbes are the cornerstone for producing biomolecules through their inherent metabolism, fast growth, and ease of scale-up. Still, there is a gap between lab scale synthesis and industrial-level production of biomolecules. The primary hurdle in commercializing the biomolecules from microbes is their economical production. Thus, strain improvement for enhanced and improved biomolecule production has gained significant interest worldwide in the past decades. Strain improvement dramatically alters the metabolic pathway, enzyme production and affects the enzyme properties, ultimately lead to the desired production. The primary approach for strain improvement is mutagenesis, genetic engineering, and genome engineering. Amongst these, random mutagenesis is very simple, efficient, and required no prior genomic knowledge, though its labor intensive and time consuming. However, this can be overpowered with a strong selective pressure. Strain improvement techniques as discussed in this review were implemented in the present study aiming to increase the production of biomolecules i.e., PHB and violacein pigment using psychotropic bacterium *Iodobacter* sp. PCH194 was isolated from high-altitude Himalayan niches. Further, efforts are to enhance the growth temperature of the bacterium for making the bioprocess more economical and sustainable for industrial production.

3. MATERIALS AND METHODS

3.1 Bacterial strain

Bacterial strain *Iodobacter* sp. PCH194 was already isolated at Molecular and Microbial Genetics laboratory, CSIR-IHBT, Palampur, India. Originally, the strain PCH194 was isolated from a sediment sample of a kettle lake situated at high-altitude region (4200 meters above sea level) in Himachal Pradesh, India. The strain was already characterized and studied for its potential to co-produce PHA and violacein pigment (Kumar et al. 2018; 2021).

3.2 Preparation of bacterial culture

Bacterial isolate *Iodobacter* sp. PCH194 from glycerol stock was streaked on nutrient agar (NA, Himedia, India) plate and kept at 20°C for 48 to 72 h until single isolated colonies were observed. Single colony from NA plate was inoculated in 100 ml flask with 10 ml nutrient broth (NB, Himedia, India) media and was incubated at 20°C, 150 rpm for 24 h. Composition of media is listed in Table 5.

Table 3.1: Composition of Nutrient Agar media.

Nutrient Agar	
Components	**mg/ml**
Peptone	5.0
Sodium Chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	20

3.3 Strain maintenance and storage

The strains were maintained at 4°C on NA plate. Sub-culturing was carried out once a month by taking a loop full of culture and streaking it onto NA plate. This was performed to maintain pure culture and avoid any possible contamination. The strains were cryopreserved in 30% glycerol at - 80°C.

3.4 Sterilization of the media and glass wares

Media used for various experiments were sterilized in flasks/tubes plugged with non-absorbent cotton at 121°C and 15 lbs steam pressure for 15-20 min. Heat sensitive media components were sterilized with 0.22 µm filters. Glass wares were sealed with autoclavable polypropylene bags before sterilization.

3.5 Standardization of mutagen concentration

The mutagens used were ethyl methane sulfonate (EMS, Sigma Aldrich, USA), methyl methane sulfonate (MMS, Sigma Aldrich, USA), and nitroso methyl urea (NMU, Sigma Aldrich, USA). All the stock solutions were prepared with 10 mg/ml concentration. They were dissolved in Milli-Q water and filter sterilized. The stock solutions were further diluted to required concentrations using the formula given below:

$$\mathbf{N_1 V_1 = N_2 V_2}$$

Where, N_1 = Normality of stock solution

V_1= Volume of stock solution.

N_2 = Normality of desired solution.

V_2= Volume of desired solution

Final working concentrations of mutagens used were 50, 100, 120, 150, 500 and 1000 µg/ml.

3.5.1 Preparation of bacterial cells for mutagenesis

The growth of bacterial culture was monitored at absorbance of 460 nm using microplate spectrophotometer (Synergy, BioTek, USA). 1ml of cell culture with OD_{460} 0.8-1.0 was transferred to 1.5 ml autoclaved micro centrifuge tubes (MCTs) and were centrifuged at 5000 g for 5 min. The supernatant was discarded and pellet was suspended in 1ml of normal saline (0.8% NaCl) to make homogenous suspension.

Different concentrations of chemical mutagens were added to the bacterial suspensions and further incubated at 20°C for 3 h with shaking (150 rpm). After 3 h, the bacterial suspensions were again centrifuged at 5000 g and the supernatant was carefully discarded. The pellet was washed twice with normal saline to remove traces of mutagen. Finally, the pellet was suspended in normal saline and serial dilutions (10^{-2}, 10^{-4} and 10^{-6}) were made. 100 µl of appropriate dilutions was spread on NA plates and incubated at 20, 25, and 28°C for two days, until visible colonies observed.

3.5.2 Standardization of physical mutation

Physical mutagenesis was done by irradiation of bacterial culture using UV rays for various time intervals. Cells from fresh bacterial culture (OD_{460} 0.8-1.0) were harvested and transferred to sterilized MCTs. The tubes were centrifuged at 5000 g for 5 min and supernatant was discarded. Pellet obtained was suspended in 1 ml of normal saline to make homogenous suspension. The suspension was spread on NA plates, and kept in UV chamber with 30 Jm^{-2} intensity of UV radiation. The plates were exposed for different time intervals ranging from 30 secs to 10 min, with UV dosage ranging from 15 to 300 Jm^{-2}min. The plates were incubated at 25, and 28°C for two days until colonies were visible. UV dosage of the bacterial cells was calculated using the following formula:

UV dose = UV intensity × time

3.6 Screening of mutants for enhanced production of biomolecules at 20^{o}C

The mutant strains were inoculated in different 125 ml volumetric flasks containing 10 ml NB each and were further used as inoculum for production media. The seed flasks were kept at 20°C for 42-48 h until OD_{460} reached 1.5 to 2.0. 1 ml of seed culture was added to 250 ml flasks containing 50 ml of production media. The flasks were incubated at 20°C for a period of four days and were harvested after every 24 h time interval.

3.6.1 Determination of biomass or dry cell weight

Biomass was routinely quantified gravimetrically (Williamson and Wilkinson, 1958). To determine the dry cell weight, at the end of respective incubation time period, 2 ml of culture broth was transferred to pre-weighed MCTs and centrifuged at 5000 *g* for

10 min. The cell pellet was dried at 60°C in hot air oven. The dry cell weight (DCW) was used as a reference for the dried PHA weight using following formula:

DCW (mg/ml) = Amount of biomass (mg)/ml

3.6.2 Extraction and quantification of violacein

At various time intervals, the cultures were centrifuged (5000 g, 10 min) to obtain cell pellet whereas the supernatant was discarded. 2 ml ethanol was added to the pellet obtained for extraction of pigment. The suspension formed was centrifuged (5000 *g*, 5 min) and the pigment was extracted, leaving the colorless pellet behind. The absorbance of the pigment was checked at OD_{570} and pellet was used for PHA analysis. The violacein pigment production was estimated using the following formula:

Violacein (mg/ml) = OD_{570} × 1.2 (Violacein standard curve factor) × Volume of pigment extracted (ml)

3.6.2 Extraction and quantification of PHA

The pellet after violacein extraction was resuspended in sodium hypochlorite and chloroform (1:1) and kept at 37°C overnight. Next, on formation of two layers, the lower layer containing PHA was pipetted out to another MCT. Chilled methanol was added to precipitate PHA and was further kept at -20°C for increased precipitation. After 15 to 25 min PHA granules were observed. The tubes were centrifuged and methanol was carefully pipetted out, leaving the PHA granules behind. PHA granules were left undisturbed for complete drying and their yield was calculated using the following formula:

PHA yield (mg/ml) = Amount of PHA formed (mg) / Volume of culture broth (ml)

3.7 Growth profile study of wild and mutant bacteria

Fresh bacterial cultures of wild and mutant strains were prepared and used as seed culture to study growth profile. 250 ml flasks containing 50 ml of NB were inoculated with 1ml of the bacterial cultures (with OD_{460} 1.5-2.0) and kept at 25, and 28°C for 33 h. Their absorbance (OD_{460}) was checked at 3 h interval for estimation of their overall growth.

For quantification of bacterial growth, three 250 ml flasks containing 50 ml NB were inoculated with the mutant strain as previously described and were kept at three different temperatures *viz.* 20, 25, and 28°C for 6 h. Before incubation 100 µl of bacterial culture was taken as control and spread on NA plate. After 6 h 100 µl from each flask was taken and spread on NA plate. All the plates were kept at 20°C for two days and their growth was compared.

3.8 Effect of thermo protectants on growth of mutant

The thermo protectants used were glycine betaine (Sigma Aldrich, USA) and glutamate (Sigma Aldrich, USA) with concentration of stock solution, 100 mM and 1 M respectively.

3.8.1 Effect of glycine betaine

Bacterial culture of mutant was grown in 250 ml flasks containing 50 ml NB. Different concentrations of glycine betaine (0, 100, 300, 600, and 900 ⌈M) were added to the flasks and kept at 28°C for incubation. Growth of mutants was monitored at OD_{460} for 56 h and was compared for different concentrations of glycine betaine.

3.8.2 Effect of glutamate

Bacterial cultures of mutant were prepared and used as inoculum. Different concentrations (1, 10, 50, and 100 mM) of glutamate were added to the flasks; with initially one flask kept as a control. The flasks were further inoculated with the bacterial culture and were incubated at 28°C. Growth of mutants was monitored at OD_{460} for 56 h and were compared for different concentrations of glutamate.

4. RESULTS AND DISCUSSION

4.1 Bacterial growth

In the present study, the bacterial strain PCH194 was used for all experiments. The bacterium can grow well at a low temperature of 4°C with optimum growth at 20°C and co-produces PHB and violacein pigment (Kumar et al. 2021). However, it was noticed that bacterium hardly grow at 25°C, and no growth was observed at 28°C and above temperature. Bioprocess using the strain for co-production of PHB and violacein pigment required low temperature for growth, which is energy demanding and can add to its production cost. Therefore, strain improvement of PCH194 is sought to enhance the growth optima toward ambient temperature while improving the yields for PHA and violacein pigment.

4.2 Generation of mutants using chemical and physical mutagens

Random mutagenesis i.e., physical and chemical mutagenesis were applied to enhance the growth temperature and production of PHA and violacein of *Iodobacter* sp. PCH194. Therefore, the strain was treated with chemical mutagens i.e., nitroso methyl urea (NMU), ethyl methane sulfonate (EMS), and methyl methane sulfonate (MMS), and physical mutagens i.e., the various dosage of UV radiation. In the literature, earlier studies have also reported mutant generation by treating microbes with physical or chemical mutagens (Brown 2000; Kumar et al. 2015).

4.2.1 Tolerance of bacterial strain PCH194 against chemical mutagen

Before proceeding to mutant generation, a lethal dose of mutagen for the bacterial survival was evaluated. For this purpose, the bacterial isolate PCH194 was treated with different concentrations of mutagen NMU, i.e., 100, 500 and 1000 µg/ml. The treated culture was spread on NA plates and was incubated at 20°C for two-three days. Colonies were observed at a concentration of 100 µg/ml at 20°C along with positive growth in the control for the different dilutions spread on the plates. Whereas no colonies were found at higher mutagen concentrations of 500 and 1000 µg/ml (Table 4). Therefore, for further standardization and treatment, mutagen concentration of 100 µg/ml was used.

Table 4.1: Presence (+) or absence (-) of bacterial colonies at 20°C with respect to different dilutions and varying concentrations of mutagen NMU used.

Conc. (µg/ml)	Dilutions			
	10^{-0}	10^{-2}	10^{-4}	10^{-6}
0	+	+	+	+
100	+	+	+	+
500	-	-	-	-
1000	-	-	-	-

4.2.2 Optimization of different dilutions for countable colonies

Optimization of serial dilutions was carried out to obtain evenly spread and countable colonies to assess the lethal effect of the mutagen on bacterial culture. Two sets of the bacterial culture were prepared. The first set was treated with NMU (100 µg/ml) for 3h at 20°C, whereas the NMU untreated second set was kept as a control. Both the sets were diluted to different dilutions (10^{-0}, 10^{-2}, 10^{-4}, and 10^{-6}), evenly spread on NA plates. The plates were further incubated at 20°C for two-three days until good growth of colonies were observed. It was found that plates with 10^{-4} dilution were easily countable (Figure 4.1). Hence, the 10^{-4} dilution was chosen for further experiments on treatment with different mutagens.

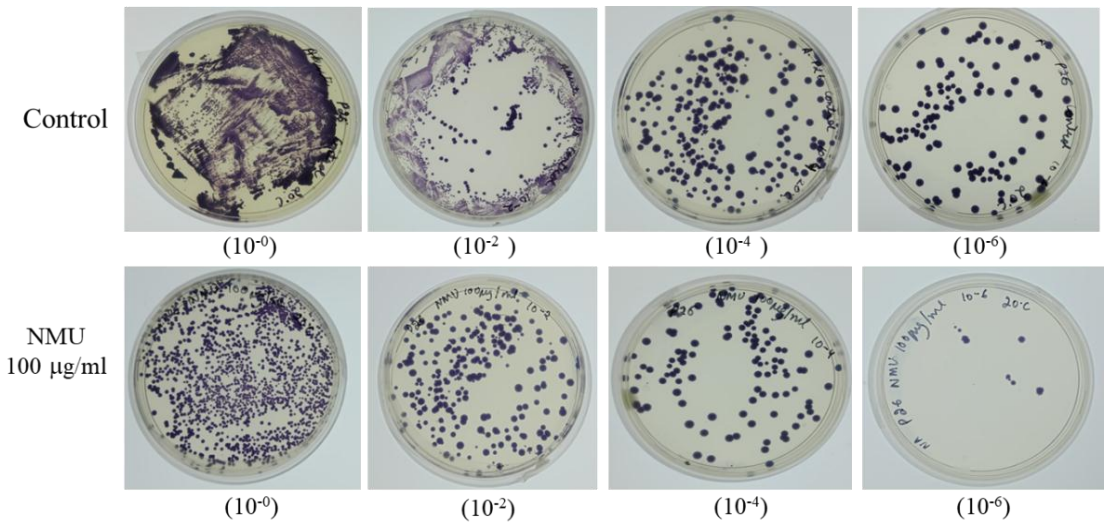

Figure 4.1: The serial dilution of bacterial culture treated with mutagen (NMU 100 µg/ml) along with untreated culture (control).

4.2.3 Optimization of chemical mutagens and their concentrations

Based on a previous experiment of mutagen lethality on a bacterium PCH194, three mutagens (NMU, EMS, and MMS) with different concentrations (50, 100, and 150 µg/ml) and a serial dilution of 10^{-4} were used. The suspensions were evenly spread on NA plates and kept at 20, and 28°C for two-three days until a single colony were observed. It was found that at a concentration of 150 µg/ml, the number of colonies were reduced to near half of the untreated cells (Table 5). There was no colony observed at 28°C, which might be due to a drastic shift in temperature for isolate PCH 194 that grows optimally at 20°C. Therefore, chose a comparatively lower temperature (25°C) and lower mutagen concentrations of 100 and 120 µg/ml for further experiments.

Table 4.2: The number of bacterial colonies obtained for PCH194 at different concentrations of mutagens on different temperatures.

	20°C			**28°C**		
Mutagen/ Conc. (µg/ml)	NMU	MMS	EMS	NMU	MMS	EMS
0	442	398	438	-	-	-
50	273	291	318	-	-	-
100	221	215	234	-	-	-
150	198	187	209	-	-	-

Note: -, indicates absence of bacterial colonies.

The bacterial cultures were treated with three different mutagens (NMU, EMS, and MMS) at concentrations 100, and 120 µg/ml and incubated for 3 h at 20°C. The suspensions were further spread on NA plates and were kept at 20 and 25°C for two days. At 20°C, many colonies were observed (Table 6) and a few colonies were found on treatment of mutagen NMU (120 µg/ml) at 25°C (Figure 4.2). On treatment of PCH194 with other mutagens (EMS and MMS), we could not obtain any colony on the plates. Hence, mutants obtained with NMU were further re-streaked on the NA plate and incubated at 25°C. The mutant colonies were successfully regrown at 25°C with subculturing. Therefore, finally, five colonies from the above experiment were selected as mutants of PCH194 and were denoted as IN1, IN2, IN3, IN4, and IN5.

Table 4.3: The number of bacterial colonies obtained for PCH194 at different concentrations of mutagens on different temperatures.

	20°C			**25°C**		
Mutagen/ Conc. (µg/ml)	NMU	MMS	EMS	NMU	MMS	EMS
0	447	447	447	-	-	-
100	221	214	199	-	-	-
120	201	212	178	7	-	-

Note: -, indicates absence of bacterial colonies.

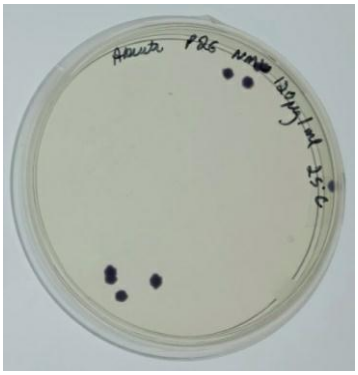

Figure 4.2: Generation of bacterial mutants at 25°C by mutagen NMU (120 µg/ml).

4.2.4 Generation of mutant using physical mutagen

Our study's prime focus was to increase the growth temperature from 20 to 25 and then to 28°C. Therefore, the mutants generated with chemical mutagens were treated again with the physical mutagen. The physical mutagens are mainly different types of irradiations i.e., X-ray, gamma rays, and ultraviolet rays. UV rays are comparatively easier to handle and less harmful than non-ionizing radiations such as X-rays. Therefore, we used UV rays as a physical mutagen for further experiments. The bacterial culture was spread over NA plates and exposed to UV radiation for different time intervals i.e., 2.5, 5, and 10 min that produces a varying dose of 75, 150, and 300 Jm^{-2}minof UV, respectively. A control plate (no UV treatment) was also processed following a similar procedure. The plates were incubated at 25, and 28°C temperature. At 25°C very slow-growing colony was observed, whereas no colony formation was observed at 28°C (Figure 4.3).

From the above experiments, it was realized that the dose of UV was high. Therefore, a time of UV exposer was reduced to 1, 2, and 3 min corresponding to 30, 60, and 90 Jm^{-2}min, respectively. Comparatively, found a visible number of colonies at 25°C, but no colony formation was observed at 28°C (Figure 4.4). A very high UV dose causes the cell death; therefore, no mutant was generated at 28°C. On the other hand, upon low UV dose treatment, the mutants obtained at 25°C were grow on a higher temperature of 28°C. Since, *Iodobacter* sp. PCH194 is an innate psychotroph and inhabitant of high-altitude facing higher UV radiations; thus, probably have an inherent tolerance to UV. Microbes of higher altitudes are established for the UV-induced DNA damage repair systems (Hirsch et al. 2004; Obertegger et al. 2011; Maayer et al. 2014; Dziewit and Bartosik 2014). Psychrophiles/psychrotrophs contains numerous genes for UV tolerance and other features such as biofilm were conferring UV tolerance (Dzewit et al. 2013; Dziewit and Bartosik 2014; Elasri and Miller 1999; Carvalho 2017; Yin et al. 2019). Therefore, UV-based mutagenesis of the bacterial strain PCH194 did not show improvement in the present study growth's temperature.

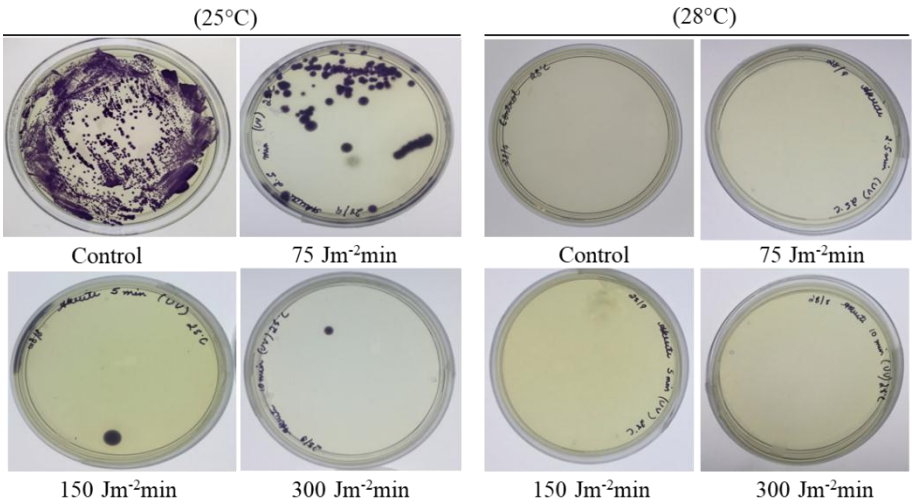

Figure 4.3: Comparison of bacterial growth in untreated (control) and treated with varying UV dosages.

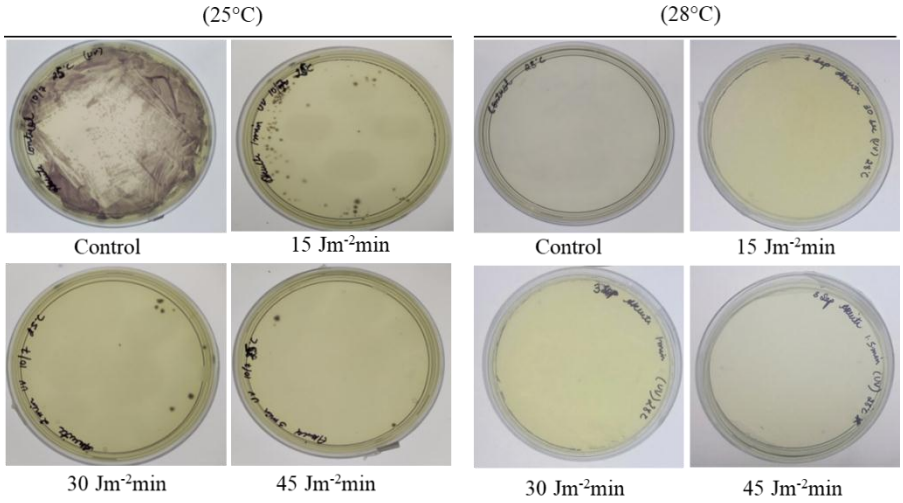

Figure 4.4: Comparison of bacterial growth in untreated (control) and treated with varying UV dosages.

4.3 Screening for enhanced production of biomolecules at 20°C

More than 2000 mutants were generated after treatment with mutagens. Finally, five mutants (IN1, IN2, IN3, IN4, and IN5) were selected on the basis of their ability to grow at 25°C compared to wild type PCH194 that grows at 20°C. Simultaneous production of PHB and violacein was carried out in 50 ml shake flask using the NB in the presence of 1.0 % glucose. The production media was inoculated with seed culture and kept at 20°C for 96 h. Samples from the production media were extracted at time interval of 24 h and used to estimate violacein and PHA. The mutants were screened for growth and biomolecule production using the wild-type strain as the reference. All mutants showed a higher amount of PHA and violacein pigment production than the wild type strain. The increased yield for PHA and violacein are discussed in the following subheads.

4.3.1 Screening of mutants for PHA synthesis

The mutants and wild-type PCH194 were screened for PHA synthesis at first. The mutants IN1, IN2, IN3, IN4, and IN5 showed production of 0.88, 0.98, 0.70, 1.12, and 1.24 mg/ml respectively; whereas wild-type strain showed 0.42 mg/ml PHA production (Figure 4.5). Among the mutants, IN5 showed highest PHA production, comparatively two times (195 %) more than the wild- type strain. Therefore, it was concluded that mutant strain IN5, IN4, and IN2 are high PHA yielding mutants generated in the current study.

Enhancing PHA production by the use of chemical and physical mutagens has been done previously. Pal et al. (2009) reported 5.4 fold increase in PHA production on treatment with UV rays in *B. thuringiensis*. Similar experiments were conducted using ethyl methane sulphonate on *C. necator* and reported 35 % increase in PHA production compared to the wild-type strain (Obruca et al. 2013). Aravind and Sangeetha (2014) studied the effects of UV radiation, acridine, and 5-bromouracil on *C. necator* and *K. intermedia*. They concluded that treatment with acridine orange is most promising, as it increases PHA production by 20, and 40 % in *C. necator* and *K. intermedia* respectively. Similarly, many other researchers have reported increased production of PHA through treatment with physical or chemical mutagens (Sreeju et al. 2011; Sangkharak and Prasertsan 2013; Bashir et al. 2014; Girdhar et al. 2014).

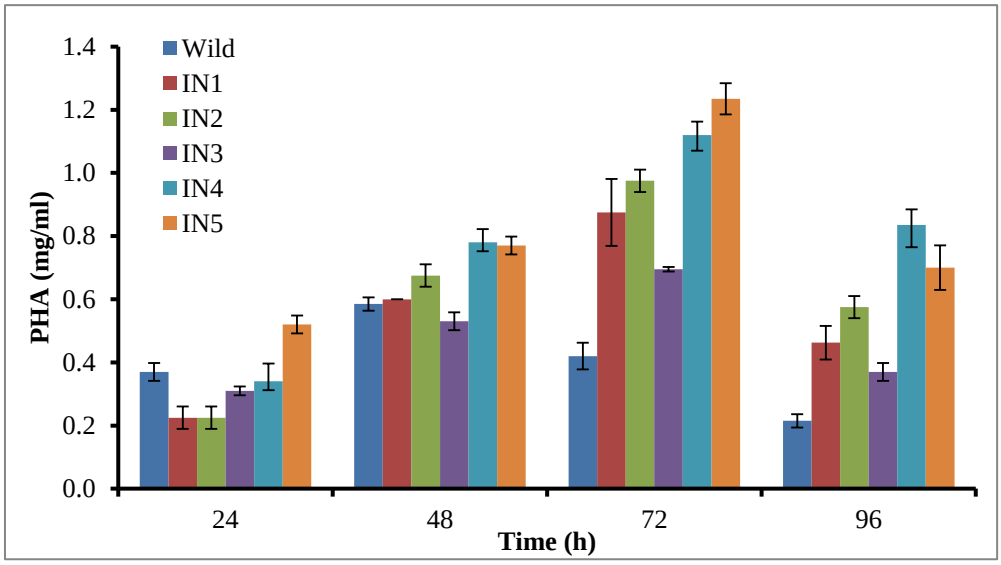

Figure 4.5: Production of PHA (mg/ml) by the mutants and wild-type strain PCH194 at different time intervals.

4.3.2 Screening of mutants for violacein pigment synthesis

Further, all selected mutant strains were evaluated for pigment production. The data obtained showed that increased pigment production at 96 h of incubation (Figure 4.6). Whereas IN1 led a higher pigment production at 72 h. earlier than 96 h. On screening for violacein, mutants IN1, IN2, IN3, IN4, and IN5 showed production of 0.88, 1.63, 0.53, 1.04 and 1.15 mg/ml, respectively. While, wild type strain showed 0.20 mg/ml production (Figure 4.6). The highest production was showed by mutant IN2 that was

eight times (715 %) better than the wild-type strain. Hence, mutant IN2, along with mutant IN5 and IN4 are the high violacein pigment yielding mutants.

A few studies have been carried out to enhance the production of pigment by the application of mutagens. One of the earliest works was done by Yongsmith et al. (1994). They demonstrated a ten-fold increase in the production of yellow pigment through the application of UV radiation. Prodigiosin production was increased eight times of the parent strain by EMS treatment (Elbialy et al. 2015). Similarly, many other researchers have used chemical mutagens combined with UV for enhancing pigment production (Issa et al. 2016; Yolmeh and Khomeiri 2016; Yolmeh et al. 2017). There are no records in the literature to enhance violacein pigment production by using chemical and physical mutagens.

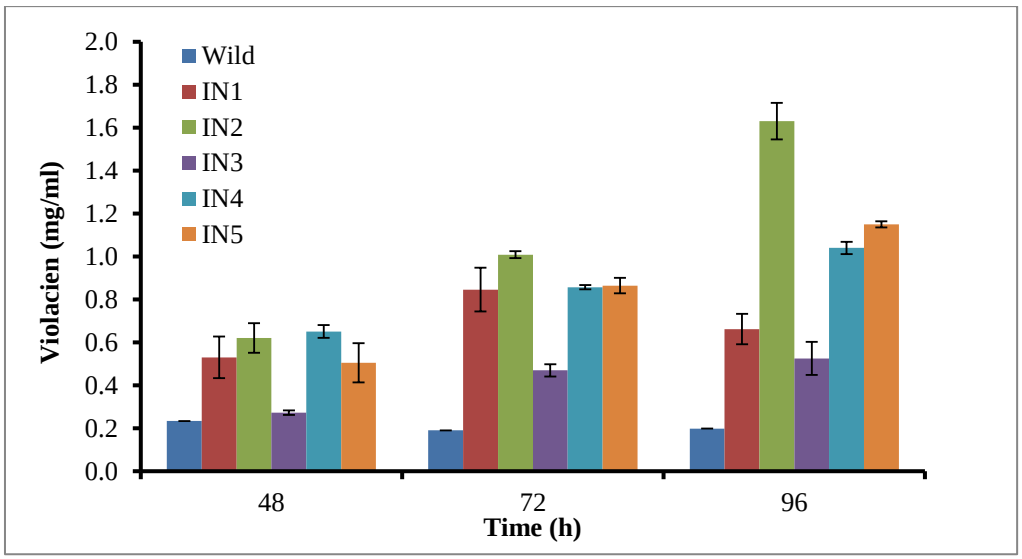

Figure 4.6: Production of violacein (mg/ml) pigment by the mutants and wild-type strain PCH194 at different time intervals.

A comparative yield of PHA and violacein production by selected mutants was summarized in Table 9. Interestingly, mutant IN4 and IN5 produced a high yield of both the biomolecules simultaneously in comparison to wild-type PCH194. It was observed that maximum production of PHA resulted after 96 h and maximum violacein production was achieved in 72 h. The major advantage inferred from the present study was the prospect of simultaneous and enhanced production of PHA and violacein through a common fermentation process. Additionally, these generated

mutants can be further re-treated with mutagens to enhance the temperature optima at 28°C without losing their high yielding capabilities.

Table 4.4: Comparison of production of PHA and violacein by the mutants and wild strain PCH194

Strains	**Biomolecules**	
	PHA (mg/ml)	**Violacein (mg/ml)**
Wild	0.42 ± 0.04	0.20 ± 0.00
Mutant IN1	0.88 ± 0.11	0.63 ± 0.07
Mutant IN2	0.98 ± 0.04	1.63 ± 0.08
Mutant IN3	0.70 ± 0.01	0.53 ± 0.08
Mutant IN4	**1.12 ± 0.04**	**1.04 ± 0.03**
Mutant IN5	**1.24 ± 0.05**	**1.15 ± 0.01**

4.4 Screening of mutants for enhanced growth at 25°C

Other than increasing the biomolecule production, we aimed to increase the growth temperature of the bacterial strain PCH194 to make them suitable for industrial fermentation. For this purpose, bacterial cultures of mutants as well as wild-type strain were kept at 25, and 28°C and their absorbance were checked at OD_{460}.

4.4.1 Screening of mutants for enhanced growth at 25°C

The absorbance of wild and mutant strains were checked at 25°C for 96 h. All the five selected mutants showed a significantly higher growth (approximately 10-fold higher) in comparison to the wild-type PCH194. Among the mutants, IN2 showed maximum increase followed by mutant IN3, IN4, IN5, and IN1 at 48 h (Figure 4.7).

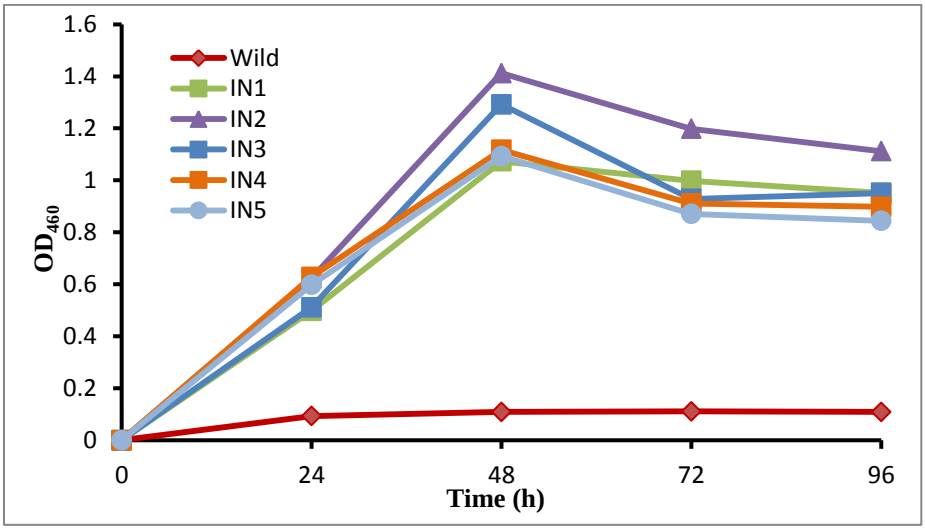

Figure 4.7: Spectroscopic absorbance data of the growth of mutants and wild-type PCH194 at 25°C.

4.4.2 Screening of mutants for enhanced growth at 28°C

At 28°C, the absorbance of wild and mutant strains was checked for a period of 30 h. Maximum growth was observed in mutant IN1 (2-fold) followed by IN4, IN5, IN2, wild-type, and IN3 (Figure 4.8). However, the growth of all mutants at 28°C was significantly lower than 25°C. The results suggested that the mutants' bacterial cells can tolerate 28°C, but with a reduced growth rate.

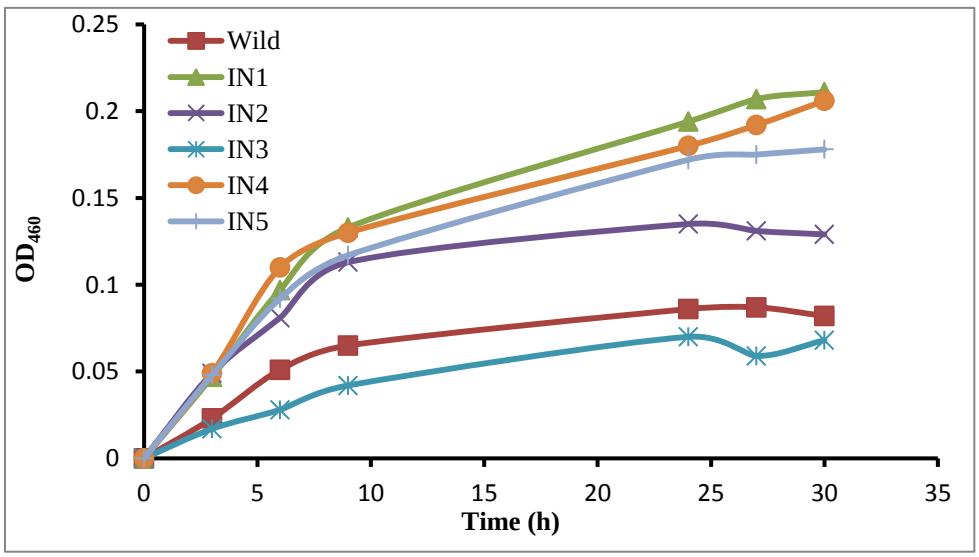

Figure 4.8: Spectroscopic data of the growth of mutants and wild-type PCH194 at 28°C.

4.4.3 Comparison of mutant cell growth at different temperatures

To confirm that the mutant (IN1) bacterial strain was able to sustain even at 28°C, a subsequent experiment was performed. Three flasks were inoculated with equal cell mass of bacterial culture grown for more than 20 h and were kept at 20, 25, and 28°C for 6 h period. Bacterial culture from each flask was taken and spread on three NA plates. Control was also kept at 20°C that has bacterial spread taken at 0 h, before incubating flasks. These plates were kept at 20°C for two days and their growth was monitored. It was observed that at 20, and 25°C the number of colonies increased, showing the multiplication of bacterial cells. However, the plate containing culture at 28°C showed almost an equal number of colonies and growth to 0 h plate (Figure 4.9). Hence, it can be concluded that the bacterial cells were alive at 28°C, but could not able to multiply.

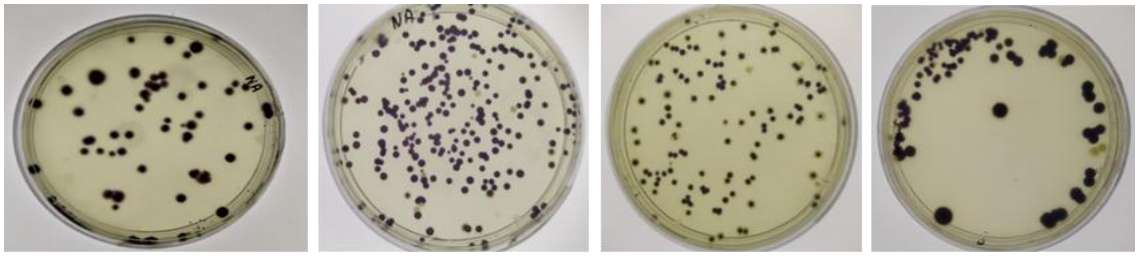

Figure 4.9: Comparison of cell growth of mutant strain IN1 at different temperatures.

4.5 Application of thermo-protectants to enhance the growth of mutants at 28°C

Glycine betaine and glutamate were found to be the most commonly used thermo-protectants for the enhancement of growth in microorganisms (Paleg 1981; Alia et al. 1998; Caldas et al. 1999; Holtmann and Bremer 2004; Adamczak et al. 2018). Therefore, we used different concentrations of glycine betaine and glutamate to enhance the growth of mutants at 28°C. Since mutant IN1 showed increase growth than other strains at 28°C, hence was selected for further experiments. NB was supplemented with different concentrations of glycine betaine *viz.*, 50, 100, 300, 600, and 900 ⌈M. This mixture was inoculated with the bacterial culture of mutant IN1 and kept at 28°C for 56 h and their absorbance was checked at OD_{460}. It was observed that there was no significant difference in the growth of IN1 in the presence of different concentrations of glycine betaine (Figure 4.10).

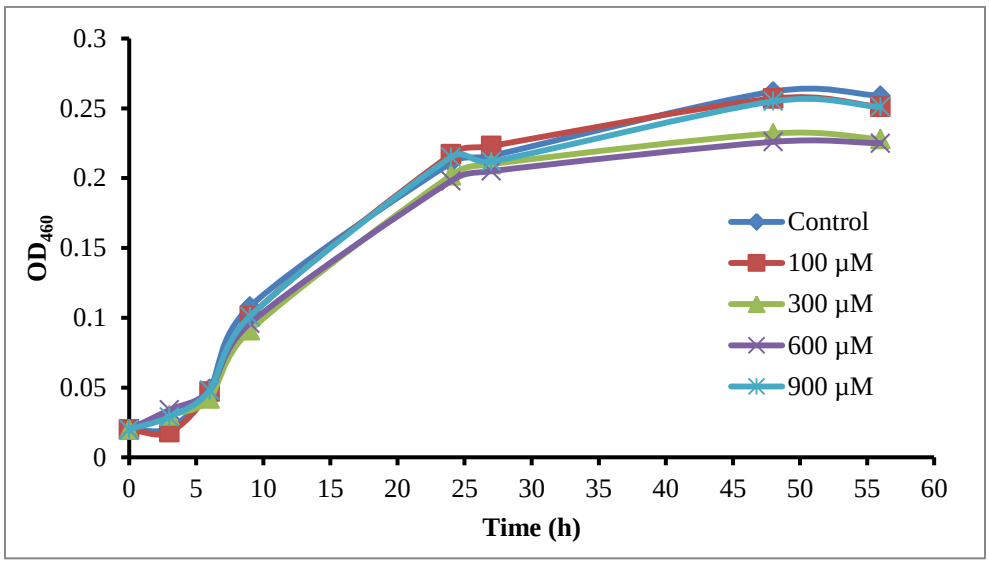

Figure 4.10: Effect of glycine betaine on the growth of mutant IN1 at 28°C.

Further, another thermoprotectant such as glutamate was added exogenously to see its impact on the growth of mutant IN1 at 28°C. The different concentrations of glutamate were used (1, 10, 50, and 100 mM) along with a control (without glutamate). The growth was observed till 56 h period and its absorbance was checked at OD_{460}. There was no significant change in the growth observed while using different concentrations of glutamate (Figure 4.11). From the above experiment, we

inferred that the thermo-protectants (glycine betaine and glutamate) used were not effective in restoring the mutant's growth ability.

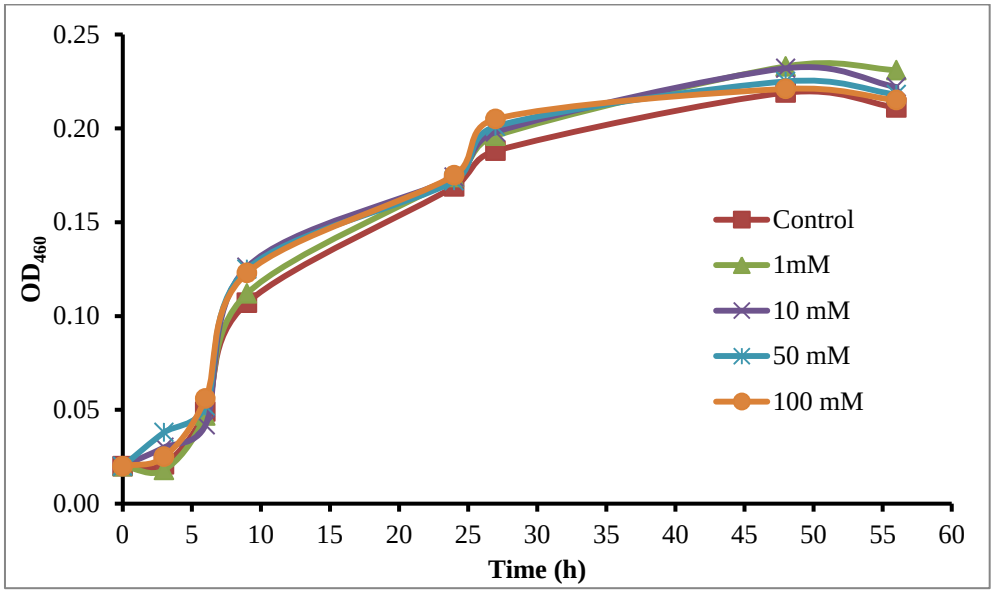

Figure 4.11: Effect of glutamate on the growth of mutant IN1 at 28°C.

5. SUMMARY AND CONCLUSIONS

Microbes are one of the largest sources of commercially important biomolecules i.e., enzymes, antibiotics, biopolymers, pigments and other chemicals are some examples. However, the industrial-scale production of biomolecules from microbes suffered from high production cost and low yields of the bioproducts. Therefore, strain improvement is inevitable for industrial biotechnology.

Our study focused on the improvement of *Iodobacter* sp. PCH194 makes it more compatible for industrial biotechnology. It increases the growth temperature and enhances the production of PHA and violacein. The physical and chemical mutagenesis method was selected for strain improvement owing to the simplicity and high efficiency. Mutants generated by chemical mutagens i.e., IN1, IN2, IN3, IN4, and IN5 showed an increase in growth temperature from 20 to 25°C. Further experiments were performed to enhance the growth temperature to 28°C using physical mutagen *viz.*, UV radiation, but no mutants were obtained at 28°C.

Mutants were screened for enhanced production of PHA and violacein pigment. The mutants IN5 and IN2 showed significantly higher PHA production and violacein as compared to wild strain (0.42 mg/ml PHA at 72 h and 0.20 mg/ml violacein at 96 h). The mutant IN5 was found to have higher PHA (1.24 mg/ml) and violacein (0.86 mg/ml) production after 72 h of incubation. Mutant IN2 showed the highest amount of violacein pigment (1.63 mg/ml) production, whereas, PHA production is comparatively higher (0.58 mg/ml). The growth of mutant IN2 was nearly ten times higher to the wild strain at 25°C. At 28°C, the growth of mutant IN1 was two times higher than wild strain. Thus, the mutants seem to be adapted to grow at 25°C, but had difficulty growing at 28°C. Thermo-protectants i.e., glycine betaine and glutamate, were used to increase and mutant IN1 at 28°C, but no significant enhancement in growth was observed.

In conclusion, the present study successfully increased the growth temperature of *Iodobacter* sp. PCH194 from 20°C to 25°C and also enhanced the production of PHA and violacein. Further studies could focus on the multiple rounds of mutagenesis to enhance the growth temperature and biomolecules production along with growth medium optimization for up-scale studies.

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