Enhanced Production of bacterial pigment possessing alpha glucosidase inhibitory activity

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Abstract- Microbial secondary metabolites have been known for their miraculous powers and have been exploited in almost all fields of human welfare. Many have a firm grip in pharmaceutical and other industries. In today's scenario, alpha glucosidase inhibitors have drawn ample scope as they have considered good candidates to treat diabetes. Understanding the importance of alpha glucosidase inhibitor and keeping in mind potentials of microbial secondary products, this research study was undertaken. The aim of this research study was to isolate pigment producing microbes and to screen out the pigments that have alpha glucosidase inhibitory activity. One bacterium that was able to produce dark maroon color pigment was isolated. A protocol was developed to completely extract the pigment. The pigment was purified by silica gel column chromatography and purity of each fraction was confirmed by performing ascending thin layer chromatography. Purified fractions obtained were tested for alpha glucosidase inhibitory activity. The pigment was found to possess good alpha glucosidase inhibitory activity. As pigment had a good activity, attempts were made to enhance the pigment production in less time. It was observed in the research process that one of the sugar alcohols has positive effect on pigment production so considering this fact, we speculated that other sugar alcohols might increase the pigment yield substantially. To check and test out speculation, we performed an experiment and our postulation turned into a fact. In presence of dulcitol the pigment production was doubled. The pigment was produced at large scale within short time by using dulcitol in the medium.

Key words- Maroon bacterial pigment, *Serratia marcescence*, Purification, sugar alcohols, alpha glucosidase inhibitor, enhanced pigment production.

Introduction-

One of the major chronic disorders is 'Diabetes mellitus' which is specified by chronic hyperglycemia. This is due to disturbances in fat, protein, and carbohydrate metabolism occurring from obstruction in insulin secretion, insulin action or both (Swagat et al., 2016). Hyperglycemia which results from complete deficiency in insulin secretion is called Type I Diabetes Mellitus (Type I DM) and which results from reduced and improper working of insulin is called Type 2 Diabetes Mellitus i.e. Type II DM (Nizam et al., 2014). Type 2 diabetes accounts for 90% to 95% of all diabetes cases, as stated by National diabetes statistic reports, 2017. Type II DM used to be frequent only in adults and therefore used to be called as "adult-onset" diabetes but in recent years its becoming more common in children. For 2017, the International Diabetes Federation (IDF) estimated the total number of adult diabetics (aged between 20 and 79 years) all over the world 425 million or thereabouts and it is presumed to increase in 2025 by 5.4% (Kim et al., 2009). Chronic hyperglycemia causes many associated disorders wherein vital organs e.g. eyes, nerves, kidneys, heart may get affected. There might be a damage, dysfunction or failure of vital organs (Andichettiar et. al., 2013). Diabetes may give rise cardiovascular diseases (Asma et. al., 2015) and atherosclerosis (Boutati et al., 2004). Diabetes also induces oxidative stress (Ceriello, 2008) which has been considered as a major pathophysiological connection between diabetes and cardiovascular diseases (Guigliano et al., 1996). Therefore there has been a need to control the diabetes by using safe and effective drugs.

Alpha glucosidase inhibitors have been considered as good candidate to combat diabetes. Alpha glucosidase inhibitors defer the rate of digestion and absorption of intestinal carbohydrate by competitively blocking glucosidase enzyme. (Maity S et al., 2016; Krentz AJ 2005)

Few glucosidase inhibitors are available commercial. Many alpha glucosidase inhibitors have been obtained from natural sources, best of them those are exploited clinically to effectively treat diabetes mellitus (to control blood sugar) are acarbose and voglibose. Most of the drugs available in the market (including acarbose and voglibose) to treat and control the diabetes pose certain side effects like diarrhea, nausea, dyspepsia, myocardial infarction, peripheral edema, dizziness (Sabjan et. al., 2012; Vishwakarma et. al., 2010; Mertes, 2001). Their tedious multistep synthesis and their association with clinical side effects have imposed a need to search new inhibitors

(Jelena B et al., 2017). Due to the importance of antidiabetic agents and their role in controlling diabetes-related mortality, the search for newer antidiabetic drugs continues.

Microbial metabolites dispense an enormous and highly diversified natural chemical reservoir from which we can explore the new drugs as potential therapeutic agents by bioactivity-targeted screenings (Elya B et al., 2011; S. H. Lam et al 2008).

In this research study, we decided to screen out bacterial and fungal pigment for their alpha glucosidase inhibitory activity because many microbial pigments have been exploited clinically as an anti-inflammatory (Ch. Romay et al., 2003), anti-carcinogenic effects (F. Chen et al., 1995; J. Schwartz et al., 1998), anti-hepatotoxic (C.A. Gallardo-Casas et al., 2010), Antioxidant (Dahah et al. 2016), antiviral (Sánchez et al.2006) agent. Prodigiosin is associated with many pharmacological activities. Prodigiosin has found to possess good and effective anticancer activity (Elahian F et al., 2012; Beatriz Montaner and Ricardo Pérez-Tomás 2003; M.C. Abrahantes-Pérez et al., 2006). This pigment has been checked on more than 60 cancer cell lines with average inhibitory concentration of 2.1 μM (Williamson et al. 2007, Manderville 2001). Prodigiosin has also reported to possess antimalarial (Singh and Shekhawat 2012, Darshan N et al., 2015), antimicrobial (Darah Ibrahim 2014, Rahul K et al., 2017) and many other important activities. Microbial pigments are present in diverse structures that create a big aspiration that many of them could match with the enzyme's substrates and could be exploited as effective natural inhibitor for enzymes that could be used for treating diseases in future. Considering the need of alpha glucosidase inhibitors and recognizing the clinical potentials of microbial pigments this research study was designed and undertaken.

Materials and Methods

Chemicals and glassware-All chemicals and glasswares were procured from renowned and acclaimed chemical and glassware manufacturers. All chemicals used for the study were of analytical grade and have given herein. All glasswares were purchased from from Scott Duran manufacturer (DURAN®, Germany). Enzyme β-Glucosidase from sweet almond, its substrate ρ- Nitrophenyl β-D-glucopyraniside(PNPG), silica mesh (200-400 mesh size),Nutrient agar, Potato dextrose agar was purchased from (Hi-Media, Mumbai,India). Na_2HPO_4,

NaH_2PO_4, Dimethyl sulfoxide (DMSO), $NaCO_3$, Acetone, Petroleum ether, methanol were purchased from Sigma-Aldrich, USA.

Isolation of Pigmented Bacteria-

For isolation of pigmented bacteria and fungi, soil and water samples were collected from oil contaminated soil and water bodies. These places were contaminated by oil industry's effluent. Samples were collected aseptically in clean and sterile plastic bottles and bags. The samples were brought into the laboratory and thereafter kept with due consideration. In case of soil samples, one gram of soil sample was blended with 10mL of distilled water and was fomented for few minutes for proper mixing. In case of water samples, 1ml of water sample was mixed with 9ml of sterile distilled water and 1mL from stock was transferred to a tube containing 9 ml of sterile distilled water and in this way samples were serially diluted up to 10^{-7}dilution. Aliquots from 10^{-6}, 10^{-7} was spread on sterile nutrient and potato dextrose agar plates. Plates were incubated at 30^0C for 48-72 hours. After every 24 hours plates were observed for the colorful pigmented colonies.

Screening for Maximum Pigment Producing Strain-

The isolated pigmented bacteria were precisely evaluated for their pigment producing ability. All isolated pigmented bacteria were grown on Nutrient agar medium, and were kept for the incubation at 30^0C for 48 hours. The bacterium that was showing intense, dazzling pigment within short incubation period was selected and was considered for the further studies.

Production of Pigment in various nutrient medium- The isolated bacterium that was selected in screening step was able to produce dark maroon intracellular (Figure 1), when present on solid nutrient medium and extracellular pigment (figure 2) when present in liquid nutrient medium. In order to determine media that favors maximum intracellular and extracellular pigment production, the isolated bacterium was grown in different media like Nutrient broth, Czapek Dox Broth, Peptone Glycerol medium, glycerol beef extract broth, Peptone water, Zobell marine broth. Bacterium was grown in liquid and solid medium. Centrifugation of broth was performed with relative centrifugal force of 3600×g for 20 minutes by using REMI research centrifuge (REMI, India). The supernatant was used to measure the pigment yield. Absorption of broth was measured at 470nm in

spectrophotometer (Elico UV-Visible double beam spectrophotometer). The uninoculated broth was used as blank in this measurement.

Extraction Of Pigment- Both extracellular and intracellular pigments were extracted by developing a suitable solvent system. Various solvent combinations were used to completely extract the pigment from broth and cells. For extraction of intracellular red pigment, 10mL acetone was put in Petri plate containing red colonies. The colored acetone solution was transferred into a beaker thereafter it was filtered to remove impurities. The colored filtrate was presumed to contain many cellular constituents and other impurities, as it was not looking glassy. To separate only pigment, that colored filtrate was taken in separatory funnel and an equal amount of n-Hexane was added in that separatory funnel. The mixture was mixed thoroughly for 2-3 minutes and then it was left undisturbed for 5 minutes. Two layers of acetone and n- Hexane were observed in separatory funnel. The n-hexane layer that had only pigment in it was removed separately into a clean beaker. The obtained layer was dried and was used as crude pigment extract.(figure 3)

Extraction of extracellular diffused pigment in broth was carried out by solvent extraction method. The broth was centrifuged with relative centrifugal force of 3600×g for 20 minutes by using REMI research centrifuge (REMI, India). The cells were separated and red colored cell free supernatant was used to extract red pigment. Supernatant was concentrated in rotary evaporator. 1mL acidified methanol (4mL HCl in 96mL methanol) was added in 5mL of concentrated supernatant and it was mixed well. Then equal amount of chloroform was added and mixture was swirled. The red pigment that had transferred into chloroform layer was separated, dried and was used as crude pigment extract.

Identification of bacterium- The isolated and selected bacterium was identified on the basis of its morphological, biochemical characters. For identification, first of all Gram staining was carried out. Bergueys manual of bacteriology was followed to identify the bacterium according to its Gram nature. Ultimately, the strain was identified and confirmed by performing the automated VITEK 2 system (Biomerieux, INC., Durham, NC, USA)

Figure 1- Intracellular pigment of *Serratia marcescence* isolated for oil contaminated soil

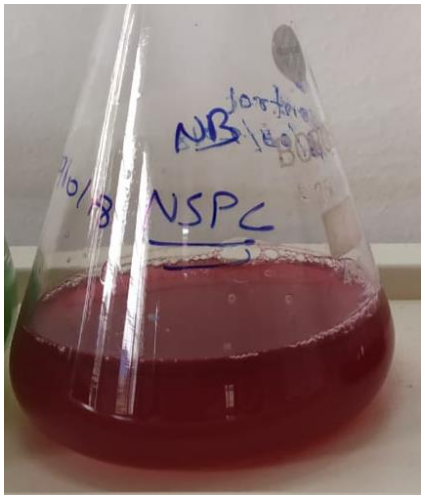

Figure 2- extracellular pigment of *Serratia marcescence*

Figure 3- Intracellular pigment of *Serratia marcescence* isolated for oil contaminated soil

Confirmation of nature of extracellular and intracellular pigment- Thin layer chromatography of intracellular and extracellular pigment was performed to confirm whether these two pigments were same or different. Ascending thin layer chromatography was carried out on TLC plates (Merck, USA). Equilibration of chromatography chamber was done by keeping solvents in that chamber for 15 minutes. The dry crude intracellular and extracellular pigment extract was dissolved in petroleum ether and both samples were spotted on TLC plate. For TLC, ethyl acetate: Petroleum ether (7:3), solvent system was used. After complete drying of sample spot, the plate was kept in equilibrated chamber with sample and was allowed to run. The plate was removed from the chamber and was observed under short UV, Long UV and visible lights.

Presumptive test for prodigiosin- As the pigment was red in color and was showing peculiar Prodigiosin like characters, we performed a test to confirm the type of pigment. A method of Gerber and Lechevalier, 1976 was followed. 5ml of extracted pigment was taken in two test tubes. One tube was made alkaline and one was acidified with the help of 1N HCL and 1M NaOH. If the pigment in acidified tube turns to pink or red and in alkaline test tube turns to yellow or tan, confirms a positive presumptive test for Prodigiosin.

Purification of Pigment- Ascending thin layer chromatography was performed on TLC plates (Merck, USA) to screen and judge the number of different fractions in the crude intracellular pigment extract. Equilibration of chromatography chamber was done by keeping solvents in that chamber for 15 minutes. The dry crude sample was dissolved in petroleum ether and sample was spotted on TLC plate. For TLC, ethyl acetate: Petroleum ether (7:3), solvent system was used. After complete drying of sample spot, the plate was kept in equilibrated chamber with sample and was allowed to run. The plate was removed from the chamber and was observed under short UV, Long UV and visible lights.

The fractions were separated moreover by using Silica gel column chromatography. The crude sample was dissolved in 2-3ml of petroleum ether and it was absorbed on small quantity of silica gel (HiMedia, 200-400 mesh size). For fractionation, a vertical glass column made from borosilicate material was used. The column was cleaned well with acetone and was thoroughly dried before packing. The column was prepared by slurry method and was cautiously packed. Sample was loaded on top of the column (450mm in length × 18mm in diameter). The fractions were eluted with gradient elution method. First, column was run 2-3 times with Petroleum ether. Then a mixture of Petroleun ether and ethyl acetate (80:20) was used to elute the compounds. Amount of ethyl acetate was slowly increased (60:40, 40:60, 20:80) and 100% ethyl acetate was used to get fractions separated without mixing. To elute the compounds which were still retained to column, possibly polar in nature, acetone and methanol (80:20) solvent system was used. The methanol was slowly increased like (60:40, 40:60, 20:80) and at last pure methanol was used to elute a compound that was retained to column.

Alpha glucosidase inhibitory activity- All the three purified fractions (SN1, SN2 and SN3) of a pigment were tested for alpha glucosidase activity. The inhibitory activity of compounds was estimated by following method of Matsui et al., 1996 but some changes were adopted. For this assay, purified alpha glucosidase enzyme

(purchased from HiMedia, India) and its substrate ρ- Nitrophenyl β-D-glucopyraniside (purchased from HiMedia, India) were used.

To perform this assay, the alpha glucosidase enzyme solution and its substrate was set at 0.5unit/ml and 5mM respectively in 0.1M phosphate buffer. A reaction mixture was made by adding 500µL of enzyme, 2ml of phosphate buffer (01.M) and 400 µL of test sample. The mixture was shaken well and was incubated at 37^0C for 30 minutes. After incubation, 500 µL of substrate was used added in the reaction system and the system was again incubated for 20 minutes. Thereafter, 2ml of 0.2M sodium carbonate was added to stop the enzymatic reaction. Sample compounds were prepared in DMSO at concentration of 1mg/mL. Acarbose was used as positive control for this inhibition assay. For every sample compound, a sample, blank, control and positive control was prepared. Blank tubes were prepared without addition of enzyme alpha glucosidase and with test compound, phosphate buffer, substrate and sodium carbonate. Control tubes were prepared by adding enzyme, phosphate buffer, substrate and sodium carbonate without test compounds. In sample tube, compound that had to be tested were added with all other components whereas in positive control, acarbose was added instead of sample compounds. Absorbance of blank, positive control, sample, and control systems were recorded at 405nm. The percentage of alpha glucosidase inhibition was calculated by following formula.

$$\textbf{Percent inhibition}(\%) = \frac{[\boldsymbol{A405\ Control - A_{405\ Sample})}]}{\boldsymbol{OD_{620}}} \times \mathbf{100}$$

Enhanced production of Prodigiosin with sugar alcohols- In our research study, it was noted that the Prodigiosin production under glycerol had increased considerably. This observation made us to speculate that other sugar alcohols might also support enhanced pigment production. For proving our speculation, and to know whether sugar alcohols support enhanced pigment production or not we performed an experiment. It was presumed that sugar alcohol might increase the pigment yield. To check the effect of sugar alcohols and to find out most effective sugar alcohol for highest Prodigiosin production, we set this experiment. We used Nutrient broth (HiMedia) with composition- Peptone 10g/L, Beef extract 10 g/L, Sodium chloride 5 g/L and Peptone glycerol broth with composition- Meat extract 10 g/L, Peptone 10g/L, Glycerol 10mL/L. Glycerol, Mannitol, Sorbitol, Dulcitol, Adonitol, Inositol, Xylitol sugar alcohols were used for the experiment. In peptone glycerol

broth, we substituted glycerol with all selected sugar alcohols in separate flasks. In nutrient broth, we added 1% of each sugar alcohol in a flask. All these flasks were inoculated with equal 5ml of fresh well grown culture media. Un-inoculated medium was kept as controls. All flasks were incubated for 72 hours at 30^0C. these two medium were used to confirm only sugar alcohols are responsible for increased yield or other media components also play a role in enhancement.

Estimation of prodigiosin production- The produced prodigiosin in each flask was quantified by measuring the absorption at 470 nm which was the absorption maxima of produced pigment. The red colored broth was centrifuged with relative centrifugal force of 3600×g for 20 minutes. The supernatant was used to estimate the prodigiosin production.

Isolation and screening of pigment producing strains-

In isolation, many bacteria with pink, yellow, orange, red pigments were isolated. In screening, the most intense, tempting colony with faster growth rate was selected for the studies. A bacterial colony that appeared after only 24 hours on nutrient agar plate that was dark maroon in color was selected for the studies. The bacterium was producing an exceptional, captivating pigment in quite less time. Other pigment producing bacteria were also producing good pigments but they were slow in growth pigment production rate hence they were not considered for the present study.

Production of Pigment in various nutrient medium-

The isolated bacterium was producing very intense and fast pigment in Peptone Glycerol medium, glycerol beef extract medium. Both intarcellular and extracellular pigment was produced maximum when this medium was used. Pigment production in Czapek Dox medium was also comparable (Table I).

Medium used	Nutrient broth	Czapek Dox Broth	Peptone Glycerol medium	glycerol beef extract broth	Zobell marine broth	Peptone water	Luria Bertani broth
Absorption at 470nm	1.964±0.01	1.915±0.01	2.405±0.04	2.248±0.07	1.633±0.06	1.787±0.06	1.606±0.03

Table I. Prodigiosin production in various medium.

Identification of bacterium-

The isolated bacterium was identifies as *Serratia marcescence* on the basis of morphological, biochemical characters and by performing the automated VITEK 2 system analysis.

Confirmation of nature of extracellular and intracellular pigment- In thin layer chromatography, it was detected that both inrtracellular and extracellular pigment were same as all the fractions and spots were obtained at a same place on chromatogram.

Presumptive test for prodigiosin- Although bacterium was identified as *Serratia marcescence*,and this bacterium is known to produce prodigiosin pigment, we performed a presumptive test to confirm the type of pigment because there is always a strong possibility that bacterium might produce diverse kind of pigments irrespective of the class it belongs to.

In presumptive test, the pigment under acidic environment turned to pink and when it was in alkaline environment it turned to yellow. This showed that the pigment was prodigiosin.

Purification of Pigment-

In TLC, we observed three spots on the chromatogram. These three different compounds were separated by executing silica gel column chromatography. The first fraction was eluted with n-Hexane and ethyl acetate mixture (80:40). The compound was pink in column**.** The second compound got eluted when pure ethyl acetate was used. The compound was Pink in column. The third compound got eluted in methanol solution. We did TLC of all samples for confirming the purity of samples collected in test tubes and test tubes with same

compound were pooled together. These three compounds were named serially as SN1, SN2, SN3 and were stored in vial and were used for further analysis.

Enhanced production of Prodigiosin with sugar alcohols- Results of this experiment went in favor of our speculation. In presence of most of sugar alcohols, the pigment production was greater than the control (pigment production in absence of sugar alcohols). The pigment production was considerably increased when dulcitol was used in medium. When xylitol and inositol was used in medium, no pigment production was observed. Mannitol also does not increase the pigment yield. Dulcitol, adonitol and sorbitol supported maximum and fast pigment production (Table II). As pigment production was increased in both medium with same sugar alcohol, it also proved that other media components are not responsible for the enhancement in pigment production.

Many researchers have reported enhanced production of prodigiosin by using various media components. Anuradha et al., 2004 in her research study reported fourty fold increase in prodigiosin yield by powdered and sieved peanut seed. Esabi Basaran et al., 2015 reported increase in prodigiosin production when ram horn peptone was used in medium. Prodigiosin production was also enhanced by using tannery solid waste by C. Sumathi et al., 2014.

Medium+ sugar alcohol	NB+Mannitol	NB+sorbitol	NB+glycerol	NB+inositol	NB+dulcitol	NB+adonitol	NB+Xylitol	NB
Absorption at 470nm	1.482	3.927	2.015	No pigment	4.354	4.103	No pigment	1.964±0.01
Medium+ sugar alcohol	Peptone mannitol broth	Peptone sorbitol broth	Peptone glycerol broth	Peptone inositol broth	Peptone dulcitol broth	Peptone adonitol broth	Peptone Xylitol broth	Peptone water
Absorption at 470nm	1.648	3.954	2.405±0.04	No pigment	4.864	4.245	No pigment	1.845±0.04

Table II-effect of sugar alcohols on yield of prodigiosin.

Alpha glucosidase inhibitory activity- The compound SN1 and SN2 showed good inhibitory activity whereas the fraction SN3 didn't showed inhibitory activity. The inhibitory activity of SN1 was as good as acrbose. The

acarbose at 1mg/ml showed 60% of inhibition and SN1 purified fractions could inhibit up to 65%. The SN2 showed 40% of inhibition (Figure 4). The results designated that studied compounds could potentially be used to control postprandial hyperglycemia.

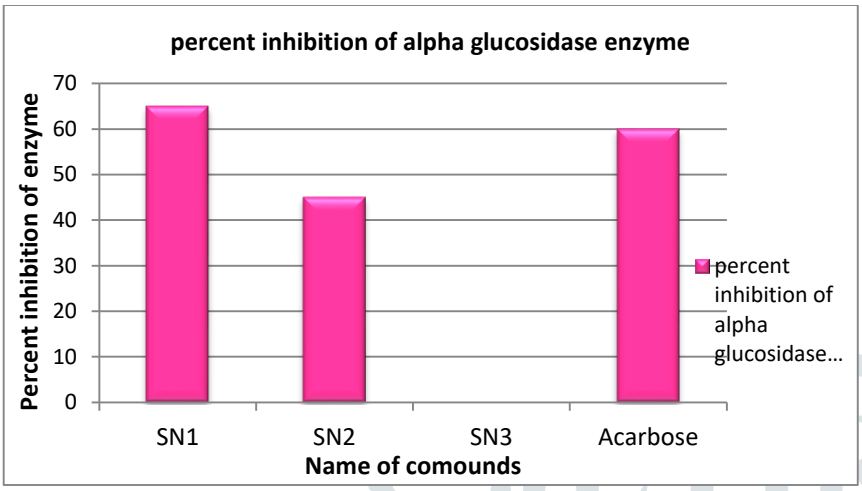

Figure 4- Graph showing percent inhibition of compounds in assay

α-Glucosidase inhibitors are good at treating noninsulin-dependent diabetes mellitus because they postpone glucose absorption in intenstine and thereby avert the increase of postprandial blood glucose level. (Gunawan-Puteri MPJ et al., 2010)

Conclusion

The presented work reported for the first time alpha glucosidase inhibitory activity of prodigiosin. The current research study underscored the ability of purified fractions of prodigiosin pigment as a potent source of alpha glucosidase inhibitor. Consequently, the degree of activity of the compound makes it a wealthy source to discover more active derivatives in on-going studies. The study also divulged about the increased production of studied pigment thereby this could be exploited in industries in future.

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