ISSN 0975 2595

PRAJÑĀ

Volume 27-28, 2019-2020

Journal of Pure and Applied Sciences

A Peer Reviewed Journal

SARDAR PATEL UNIVERSITY
VALLABH VIDYANAGAR
Gujarat - 388 120, INDIA
www.spuvvn.edu

PRAJÑĀ - Journal of Pure and Applied Sciences
(Abbreviation: *PRAJÑĀ - J. Pure & Appl. Sci.*)

Editorial Board

PRAJNA - Journal of Pure and Applied Sciences

A Peer Reviewed Journal

Volume 27-28, 2019-2020

ISSN 0975 – 2595

An Official Publication of

Sardar Patel University Vallabh Vidyanagar,

Gujarat - 388 120, India

www.spuvvn.edu E-mail: spu.prajna@gmail.com

http://spuvvn.edu/academics/publications/index.php?sphrase_id=486549

Sardar Patel University Press, Vallabh Vidyanagar, Gujarat - 388 120, INDIA

Sardar Patel University
is grateful to

Prof. Vithalbhai A. Patel

**Humboldt State University, Arcata, Califonia, USA,
whose generous donation to organize academic activities
in the name of Shri Ishwarbhai Ambalal Patel (Shertha) helped
to meet a part of the publication cost of PRAJNA Journal.**

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The Editorial Board of PRAJNA is grateful to all those experts who rendered their valuable services as referees for reviewing the papers received for this volume of the Journal.

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A Peer Reviewed Journal
Indexed in Indian Science Abstracts
Published by the Registrar, Sardar Patel University, Vallabh Vidyanagar, Gujarat - 388 120
Printed at Sardar Patel University Press, Vallabh Vidyanagar, Gujarat-388 120

PRAJÑĀ - Journal of Pure and Applied Sciences
Volume 27-28 2019-2020

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PRAJÑĀ - Journal of Pure and Applied Sciences Vol. 27-28: 01 - 11 (2019-20)
ISSN 0975 - 2595

Induction of apoptosis in *Saccharomyces cerevisiae* cells by Heliamine, an alkaloid from cactus *Backebergia militaris*

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ABSTRACT

Heliamine, an alkaloid from Backebergia militaris, was found to induce apoptosis in Saccharomyces cerevisiae cells. Heliamine induced apoptosis was preceded by increase in ROS in cells and antioxidant treatment could inhibit apoptosis. Pathway details of heliamine induced apoptosis was studied by using mutants of apoptosis inducing factor (aif1), metacaspase (yca1) and AIF over expressing strains. Heliamine treated aif 1 cells showed more growth than wild type cells and yca1 cells, indicating role of AIF in heliamine induced apoptosis. In a confirmatory experiment, the growth of similarly treated, AIF overexpressing strains was found to be reduced, ascertaining AIF induced apoptosis. Moreover, other known effects of AIF, namely induction of chromatin condensation and DNA fragmentation were also observed in agarose gel electrophoresis and DAPI staining respectively. These results confirm that heliamine induces apoptosis in S. cerevisiae cells by metacaspase independent, AIF mediated pathway.

Keywords: Heliamine; *Saccharomyces cerevisiae*; Reactive Oxygen Species; apoptosis; metacaspase 1.

INTRODUCTION

According to World Health Organization (WHO), cancer is the leading cause of death in the world after cardiovascular and respiratory diseases [1], although in current pandemic respiratory diseases predominate. Cancer is caused by mutation in one or more cell cycle regulatory genes, which results in uninhibited growth of cells resulting into tumor formation. Normal cells when mutated, initiate cell death by apoptotic pathway, however cancerous cells fail to induce apoptotic pathway. Many successful chemotherapeutic agents as well as radiation therapies employed for the treatment of various types of cancers have long been observed to induce apoptosis in transformed cells, indicating that the treatments that increases the rate of apoptosis, could be useful for the treatment of cancer [2]. Therefore, apoptosis is widely studied in cancerous as well as chemotherapy-treated as well as untreated tissues to get insight of pathogenesis of cancer. Apoptosis induced cell death is extremely useful in anti-cancerous therapy [2, 3].

Existing treatments of chemotherapy and radiotherapies have multiple long term toxic effects as they not only cause death of cancerous cells but also of normal ones. There are numerous cytotoxic compounds extracted from plants

known to induce cell death by apoptotic pathway [2]. The advantage of such natural compounds is that they have little or no effect on the normal cells. Although these plant-derived compounds are extremely useful, very few of them are being used in medicine due to their lower efficacy and lack of details of their mode of action.

S. cerevisiae is used as a model to study apoptosis since more than two decades as they offer several advantages like culturing yeast cells is methodologically simple, also yeast is a eukaryotic cell with known genome sequence [4]. *S. cerevisiae* cells used in the present studies exhibit typical markers of apoptosis and physiologically quite similar to multicellular organisms and many yeast orthologues (genes that share common ancestors) of mammalian yeast proteins have been identified [4].

Various chemicals, heavy metals and pharmacological agents have been found to induce apoptosis in yeast cells by one of the two types of pathways, one is metacaspase dependent and another is metacaspase independent pathway. Apoptosis inducing factor 1 (AIF1) induced apoptosis is metacaspase independent pathway.

In the present study, we have investigated the effective concentration and mechanism of anti-proliferative action of one of such plant derived cytotoxic alkaloid, Heliamine (6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline). Heliamine is an alkaloid, isolated from a tree-like cactus *Backebergia militaris*. Previous study of this compound on rat sarcoma 45 cells revealed that, it has anti-cancerous effects [5]. Our aim in the present studies is to understand the mechanism of cell death induced by heliamine. For this, we have studied its effect on ROS levels and role of AIF, metacaspase in inducing apoptosis and tried to develop an insight into its mechanism of action in a model organism *S. cerevisiae*.

Materials and Methods

Heliamine was procured from Sigma-aldrich and dissolved in water.

Yeast strains and Growth conditions

S. cerevisiae wild type BY4741 (*MATa, his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) was procured from MTCC, Chandigarh, India. Mutant strains of *S. cerevisiae* Δ*yca1* (MATa; ura3-52; leu2-3,112; his3-Δ1;YOR197w::kanMX4) and Δ*aif1* (MATa, his3Δ1; leu2Δ0; met15Δ0; ura3Δ0;YNR074C::kanMX4) were previously obtained as a gratis. All the cells were grown at 30°C in YPD medium containing 1% Bacto-yeast extract, 2% Bacto-peptone and 2% Dextrose [6]. Solid medium was prepared by addition of 2% agar. For each experiment, the cells were grown up to the number of 1.5 x 10^6 at 30 °C and incubated at the same condition for 24 hours.

Viability assays

Viability assays were carried out using trypan blue exclusion assay. Live cells, having intact cell membrane inhibited the passage of trypan blue

dye, were seen colorless whereas dead cells, which lost the cell membrane, were stained in blue color. The exponentially growing cells in YPD broth were treated with heliamine at different concentration ranging from 1mg/ml to 10mg/ml in 30ml cell culture media and incubated for 24 hours at 30˚C in rotary shaker (150 rpm). After the treatment, cells were harvested by centrifuging at 10,000 rpm for 5 min and washed thrice with PBS. Percentage viability was calculated from three independent experiments of trypan blue staining [7]. At least 300 cells were counted in each treatment which are compared with control one.

Acridine orange (AO) / Ethidium bromide (EtBr) staining

The co-staining of AO/EtBr is extremely useful for detection of apoptotic or necrotic cells. Acridine orange stains both live and dead cells whereas ethidium bromide stains only dead cells. The staining can evaluate the morphology of nucleus. Apoptotic cells have fragmented or condensed nuclei whereas necrotic cells resemble to viable cells in nuclear morphology. Live and early apoptotic cells appear in green color. Late apoptotic and necrotic cells appear in orange color [8]. The nucleus morphology was also evaluated using DAPI staining.

For detection of apoptosis, 24 hours heliamine treated as well untreated *S. cerevisiae* cells were evaluated using Acridine orange (AO; Sigma-Aldrich) / Ethidium bromide (EtBr; Sigma-Aldrich) staining [8]. Briefly, treated and untreated cells were harvested by centrifugation to pellet down the cells. Cells were suspended in 1 X PBS. 2µl of AO/EtBr solution (containing equal volume of 100µg/ ml AO and 100 µg/ml EtBr) was added in 20 µl of cell culture. Dye and cells were mixed properly and 10µl mixture was taken immediately for observation in fluorescence microscope. Around 300 cells were examined under fluorescence microscope using fluorescence filter at 100X objective (Olympus Bx41, Japan).

DAPI staining

For the detection of chromatin changes, the chromatin specific dye 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI; Himedia, India) was used [9]. In brief, for DAPI staining cells were harvested after the treatment for 24 hours. Then cells were washed with PBS (pH 7.4), incubated with 1 µg/ mL DAPI in PBS for 10 min at room temperature in dark. Cells were rinsed thrice with PBS and examined the change in chromatin structure under fluorescence microscope using fluorescence filter at 100X objective (Olympus Bx41, Japan).

DNA fragmentation study

DNA of 24 hours treated and untreated *S. cerevisiae* cells were isolated and electrophoresed on 0.8% agarose gel as described by Patel *et al* [10]. Briefly, treated and untreated

S. cerevisiae cells were harvested by centrifugation at 8000rpm for 5min. Supernatant was discarded and cell pellet was washed twice with PBS to remove extra impurity. Cells were resuspended in 0.5ml of distilled water in 2ml of micro-centrifuge tube. 0.2mL of lysis buffer (2% Triton X-100, 1% Sodium dodecyl sulphate, 100mM NaCl, 10 mM Tris HCl (pH-8.0), 1 mM EDTA (pH-8.0) and 0.2mL of Phenol:chloroform:isoamyl alcohol (25:24:1) were added in to cell suspension. To this acid-washed glass beads were added and vortexed for 5 min with intermittent cooling to break around 90% of the cells. DNA was separated from cell debris through by centrifugation for 5min at room temperature. Upper aqueous layer was transferred in separate tube and added with $1/10^{th}$ volume of 3M sodium acetate (pH-5.2) and 2.5 volume of 100% ethanol (chilled) to precipitate the DNA. This was spun using microfuge for 5 minutes, pellet was washed with 70% chilled alcohol, air dried and resuspended in 20-30 µl of Tris-EDTA buffer (pH-7.5). DNA was run on agarose gel electrophoresis using 0.8% agarose and Tris - acetate – EDTA buffer (pH -8.2). Image of electrophoresed agarose gel (pre-stained with ethidium bromide) was captured using ChemiDoc XRS+ (Bio Rad) instrument and analyzed by image lab software.

ROS detection

Accumulation of intracellular reactive oxygen species (ROS) were evaluated using ROS sensitive fluorescence probe 2', 7'-dichlorofluorescein diacetate (DCFDA; Sigma-Aldrich) as described earlier [11]. Exponentially growing cells were treated with heliamine (5mg/ml) for a period of 3 hours. After the treatment cells were harvested at 8000 rpm for 10 minutes and rinsed with PBS for thrice. Pellets were resuspended in PBS (0.1M, pH 7.4) and incubated with 10 µM DCFDA for 30 minutes in dark at 30°C. Stained cells were observed under fluorescence microscope (Olympus Bx41, Japan) using fluorescence filter at 40X objective.

Mutant assay

S. cerevisiae BY4741 wild-type, Δ*yca1* and Δ*aif1* cells were grown for overnight in YPD liquid media. When the cell-count reached to 1.5 x 10^6 cells/ml, it was treated with heliamine at 5mg/ml concentration for a period of 24 hours at 30°C. Untreated cells were also grown for same period of time and condition. After treatment period, cells were diluted using fresh YPD medium at different dilution i.e. 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. The 10µl of diluted cells suspension was spread on YPD agar plates to grow the survival colonies for 3 days at 30°C. The survival colonies of treated cells were compare with untreated one.

Statistics

Statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparisons test and two-way ANOVA followed by Bonferroni's multiple comparisons test. The

results represented as the mean ± SD using Graph Pad Prism 6.0 software, San Diego, USA.

RESULTS

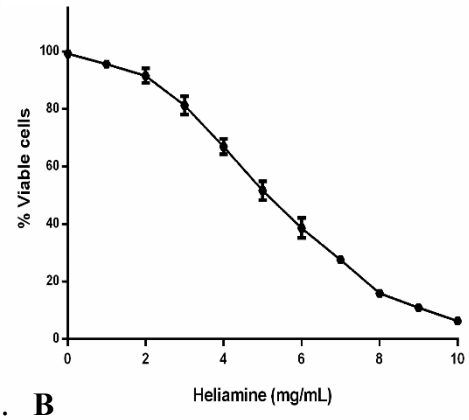
Heliamine inhibits the growth of *S. cerevisiae* cells

The effect of heliamine on growth of BY4741 wild-type *S. cerevisiae* cells was evaluated by treating with different concentration of heliamine for a period of 24 hours. A significant reduction in cell viability was noticed in heliamine treated cells in a dose dependent manner compared with control, which was treated with the same volume of extra pure water (Figure 1A). At higher concentration (20mg/mL), number of viable cells found was not significant. The IC50 concentration found from this study was 5mg/mL, which was used for further studies.

Heliamine induces the apoptosis

Whether the heliamine induced death in *S. cerevisiae* cells was by apoptosis or necrosis was confirmed using AO /EtBr dual as well as by DAPI staining. Heliamine was observed to induce apoptosis at 5mg/mL (IC_{50} concentration) (Figure 2). Compared to the control, significant number of early and late apoptotic cells were seen, whereas live cell count was decreased due to heliamine treatment (Table 1).

1. **A**



1. **B**

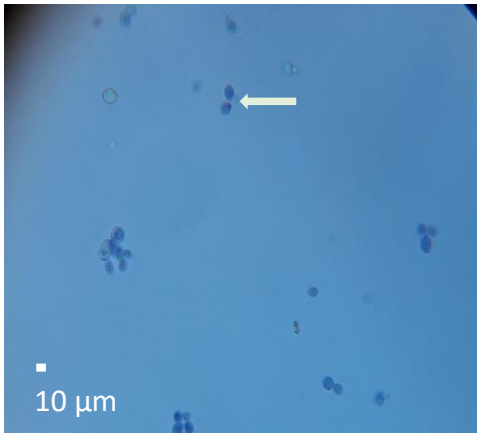


Figure 1 Heliamine inhibits the growth of *S. cerevisiae* cells. Wild type (BY4741) *S. cerevisiae* cells grown in YPD broth till 1.5 x 10^6 cells/mL cell density were treated with different concentrations of heliamine for 24 hours, cells treated with same volume of extrapure water was taken as negative control. **A.** The plot of % cell viability versus heliamine treatment was presented using trypan blue dye exclusion assay. **B.** Representative picture of trypan blue dye exclusion assay where the dead cells were stained with trypan blue, arrow indicates the dead cells.

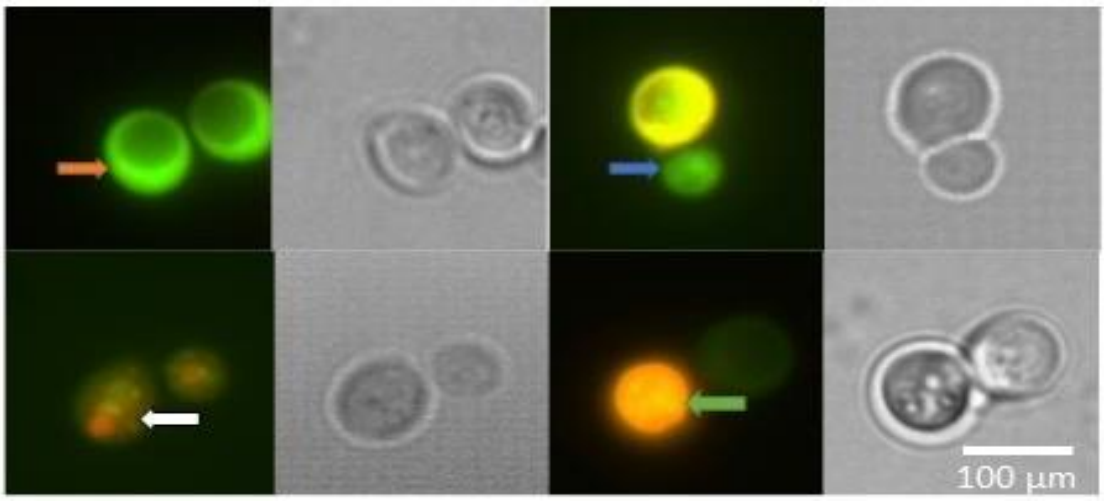
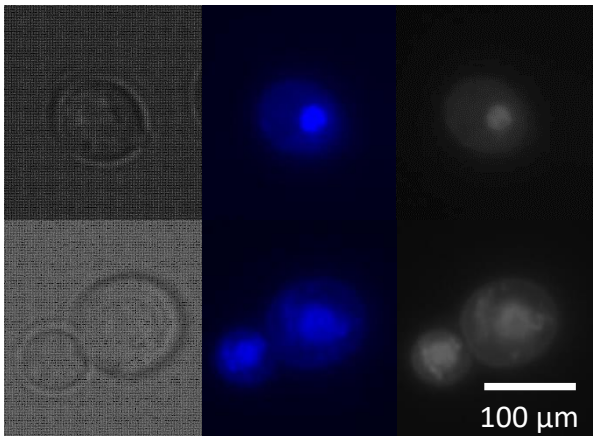


Figure 2 Acridine Orange / Ethidium bromide staining of *S. cerevisiae* cells. Cells treated with heliamine showing early apoptosis (blue arrow) as well late apoptosis (white arrow) due to condensed chromatin, whereas live cells seen in uniformly green color (red arrow). The necrotic cells were seen in orange color (green arrow).

Treatment	Live cells (in %)	Early apoptotic cells (in %)	Late apoptotic cells (in %)	Necrotic cells (in %)
Control	92 ± 3	1.6 ± 0.5	3.7± 1.7	5 ± 1
Heliamine 5mg/ml	45 ± 4.5	19 ± 5	51.57± 7.8	8.7 ± 3

Table 1 Around three hundred cells were examined from each sample for apoptosis evaluation under the fluorescent microscope at 100X objective. Each value represents the mean ± SD of triplicate determinations from three independent experiments.

3. **A**



3. B

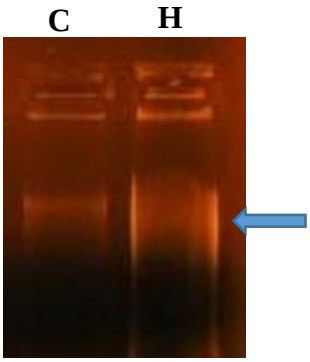


Figure 3A DAPI staining of *S. cerevisiae* cells. Cells treated with Heliamine showing condensed chromatine (lower side) compare to live cells (upper side). **3B**. Effect of heliamine on DNA integrity. Arrow indicates the degradation of DNA due to heliamine treatment. C, Control; H, Heliamine treated.

DNA condensation was also evaluated using DAPI staining. Heliamine induced the chromatin condensation at the concentration of 5mg/mL (Figure 3A). Compared to the control around 50% more condensed chromatin appeared in *S. cerevisiae* cells (Table 2).

Treatment	Chromatin condensation in yeast cells (DAPI staining)
Control	8.79 ± 1.53 %
Heliamine 5mg/ml	48.56 ± 6.3 %

Table 2 Around three hundred cells were examined from each sample for apoptosis evaluation under the fluorescent microscope at 100X objective. Each value represents the mean ± SD of triplicate determinations from three independent experiments.

Heliamine induces the ROS

Accumulation of intracellular reactive oxygen species is one of the major biochemical responses during apoptosis. The fluorescence probe DCFDA was used to detect ROS. DCFDA is converted into DCF by cellular esterase. DCF after reacting with ROS gives green fluorescence [12]. Heliamine induced more ROS generation compared to control in *S. cerevisiae* cells (Figure 4A). Around 3.5 times more ROS was produced in heliamine treated cells compared to control. Heliamine induced ROS level was decreased due to co-treatment with NAC, which suggests that NAC could control the ROS levels in *S. cerevisiae* cells.

Heliamine induces the apoptosis inducing factor 1 (*aif1*) mediated apoptosis.

Whether the induction of apoptosis by Heliamine in *S. cerevisiae* cells involved caspase dependent or caspase independent pathway was studied using metacaspase 1 (Δ*yca1*) and apoptosis inducing factor 1 (Δ*aif1*) mutant strains. Heliamine treated and untreated wild type as well as mutant cells were grown on solid media and colonies were counted. The survival of heliamine treated *aif1* mutant cells was increased compared to wild type and Δ*yca1* cells whereas aif1 overexpressed cells exhibited more susceptibility towards heliamine treatment (Figure 5). As lack of AIF resulted in increased growth but its overexpression prompted in lesser growth, this clearly indicated that apoptosis inducing factor 1 has a potent role in heliamine mediated apoptosis.

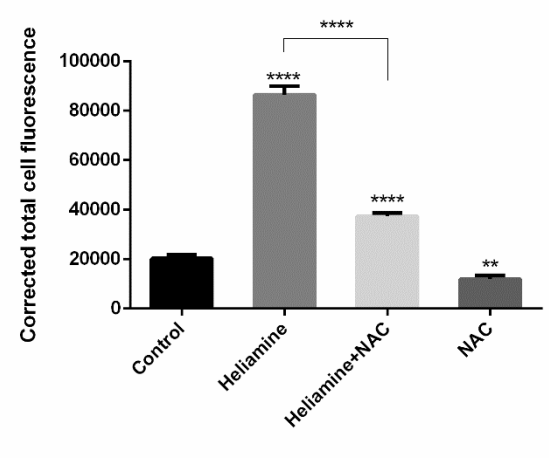


Fig 4 B The ROS induced by various treatments were quantified in the form of corrected total cell fluorescence using image J software.

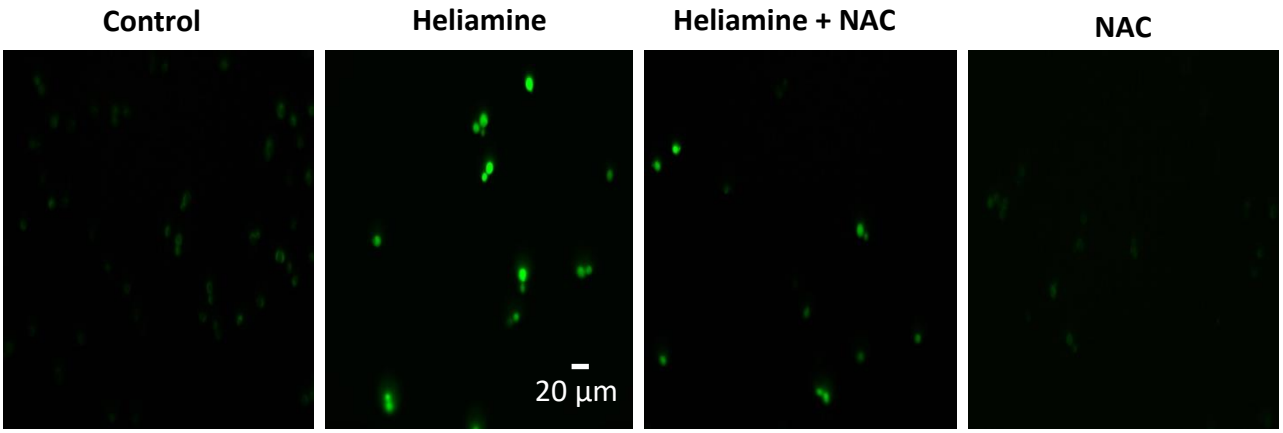


Figure. 4 A. ROS levels in *S. cerevisiae* cells treated differently were detected using DCF DA probe. Heliamine treatment significantly increased the ROS levels and co-treatment with NAC controlled it.

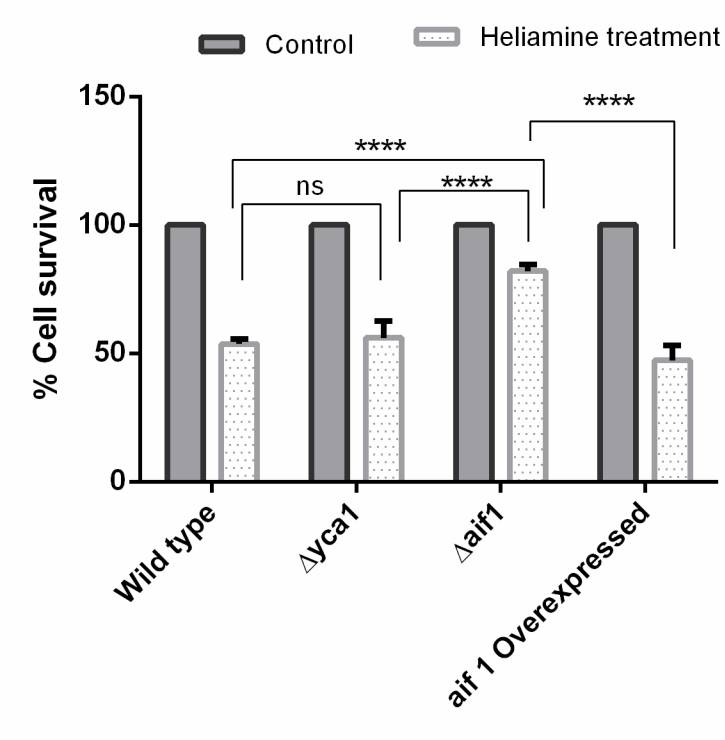


Figure 5. Heliamine induces apoptosis by activation of apoptosis inducing factor 1 in *S. cerevisiae* cells. Wild-type, metacaspase mutant (Δycal), apoptosis inducing factor 1 mutant (Δaif1) and apoptosis inducing factor 1 overexpressed of *S. cerevisiae* cells were treated with heliamine (5mg/ml) for 24 hours. After three days of treatment the number of cells survived were counted on YPD agar plats.

DISCUSSION

Heliamine exhibited dose dependent effect on *S. cerevisiae* cells. At lower concentration it did not inhibit the growth of *S. cerevisiae* cells, however with increasing concentration, it affected the viability significantly. The effective concentration range of heliamine was found to be from 1mg/mL to 10 mg/mL and its IC50 was found to be 5mg/mL (Figure 1). It is useful to find the IC50 concentration of effective compound as it directs its usefulness for medicinal usage. The IC50 concentration of heliamine found in the present studies is quite high as many effective anti-cancerous metabolites reported in the literature have effective concentrations below 1 mg. However, heliamine might be useful for simultaneous treatments with chemotherapeutic agents. Many compounds like citral (3,7-dimethyl-2,6-octadienal), curcumin ((1E,6E)-1,7-bis (4-hydroxy- 3-methoxyphenyl) -1,6-heptadiene-3,5-dione) used in the combination treatments have been found to reduce toxicity and increase the efficacy [10, 13]. Although, Heliamine is reported to have anticancer activity on rat sarcoma 45 cells [5], its IC50 concentration and mechanism of action has not been reported.

Heliamine was found to induce programmed cell death in *S. cerevisiae* cells, as confirmed by AO/EtBr staining. The common characteristics of cells undergoing apoptosis like chromatin condensation and DNA fragmentation were observed in DAPI staining and agarose gel electrophoresis results (Figures 3 A, 3 B). Many compounds at higher concentrations show undesirable effects, for example hydrogen peroxide induces apoptosis at lower concentrations but becomes necrotic at higher concentrations, but heliamine was found to induce apoptosis even at 5 mg/mL concentration.

ROS is the central molecule for apoptosis induction and regulation [5, 11]. ROS was found to be increased in heliamine treated cells and simultaneous treatment by NAC (anti-oxidant) could control the ROS levels as well as the anti-proliferative activity of heliamine (Figures 4 A, 4 B). Mechanism for induction of apoptosis by heliamine was further investigated using apoptosis pathway mutants of *S. cerevisiae*. The metacaspase mutants of *S. cerevisiae* cells were expected to grow in heliamine treated cells, as they lacked the metacaspase enzyme, however such cells showed almost similar growth as compared to heliamine treated wild type cells, which implied that metacaspase might not have any role in the heliamine induced apoptosis. The other mutant strains, *aif 1* mutants, on the contrary showed more growth in heliamine treated cells in spite of lack of apoptosis inducing factor (AIF) (Figure 5). This result clearly indicated that AIF might be required to induce apoptosis in heliamine treated *S. cerevisiae* cells. To confirm this, *aif1* overexpressing strains were employed, which upon heliamine treatment, could not grow well and confirmed induction of apoptosis in the presence of *aif1*. AIF1 is a

mitochondrial flavoprotein in the intermembrane space, important for cellular homeostasis. Upon induction of apoptosis, it gets translocated to nucleus, where it leads to chromatin condensation and DNA fragmentation [14, 15]. DNA fragmentation in yeast cells was confirmed by agarose gel electrophoresis and chromatin condensation was observed by DAPI staining. These results confirm that heliamine induces apoptosis in *S. cerevisiae* cells by caspase independent, AIF mediated pathway.

CONCLUSION

Heliamine was found to have anti-proliferative effect as it induces apoptosis in *S. cerevisiae* cells and its IC50 was found to be 5 mg/mL. The metacaspase mutant strain of *S. cerevisiae* was observed to be sensitive to heliamine treatment but *aif1* mutant was found to be resistant. The reduced growth in metacaspase mutant and increased growth in *aif1* mutant cells in heliamine treated cells, in comparison to similarly treated wild type strain, indicated that AIF might be involved inducing the apoptosis. The *aif1* overexpressing cells exhibited more susceptibility (reduced growth) in heliamine treated cells, which confirmed the role of AIF in heliamine mediated apoptosis. Based on these results it was concluded that heliamine induces ROS mediated, apoptosis inducing factor 1 dependent apoptosis in *S. cerevisiae* cells. The results found could be useful in assessing the role of heliamine in combinatorial chemotherapy treatment.

ACKNOWLEDGEMENT

Authors are thankful to P. G. Department of Biosciences, Sardar Patel University for providing research facilities.

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PRAJÑĀ - Journal of Pure and Applied Sciences Vol. 27-28: 12 - 25 (2019-20)
ISSN 0975 - 2595

Serratia marcescens promotes growth and induces systemic resistance in *Glycine max* L.

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ABSTRACT

Plant Growth Promoting Rhizobacteria, one of the best alternatives of chemical fertilizers, induce broad-spectrum, systemic resistance in plants against biotic stress. Glycine max L. is a nutritionally important legume affected by several pathogens. The Fusarium species causes severe yield loss of Soybean around the world. We report here plant growth-promoting characteristics of a bacterium (MP3), isolated from the rhizosphere of Soybean. Isolate MP3 improved the percentage of germination of uninfected as well as F. oxysporum infected Soybean seedlings from 36.66 % to 56.44%. The treatment of MP3 isolate also increased the dry weight of fungal infected plants from 0.453 g to 0.929 g and number of leaves from 12.11 to 24.36 as measured after 35 days of growth. As MP3 promoted the growth of infected plants also, elicitation of resistance was evident, which was corroborated by finding increased activities of the defense-related enzymes in germinated seedlings. MP3 was identified by biochemical and molecular characterization as Serratia marcescens.

Keywords: Serratia marcescens, Soybean plants, Fusarium oxysporum, ISR

INTRODUCTION

Soil microorganisms play important roles in biogeochemical cycles by decomposing organic matter for their nutrition acquisition and metabolism and contribute to soil fertility. In rhizospheric soil, microbes thrive as free as well as symbiotic in nature, some of which help the plants to grow better are called Plant Growth Promoting Rhizobacteria (PGPR). They are also known as Plant Health Promoting (PHPB) Bacteria (1). PGPR promotes the growth of plants by direct and indirect mechanisms. Direct mechanism includes production of phytohormones such as IAA, Gibberellins, Cytokinins, and Abscisic acid, solubilization of phosphate, potassium, zinc, etc. and fixation of atmospheric nitrogen. The indirect mechanism includes production of Siderophores, competition of nutrients, and biocontrol (inhibiting the growth of pathogen). PGPR also elicits immune systems in plants, known as Induced Systemic Resistance (ISR), which is different from the resistance developed in plants upon pathogenic attack, called Systemic Acquired Resistance (SAR), however both types of resistance activate pathogenesis-related proteins (PR proteins). Since many types of PR proteins are produced by plants, they had been initially classified into five families, PR1 to PR5, and later many were added,

up to 16 and more. Commonly expressed PR proteins in plants include PR-2, PR3 & PR4, which are hydrolytic enzymes involved in the degradation of the cell wall of pathogens like β 1-3 glucanase, Chitinase, etc. (2). The major pathways that are activated in plants for the defence purpose include Phenylpropanoid, Lipoxygenase (LOX), Sesquiterpene, Oxylipin, etc. Estimating the activities of enzymes involved in these pathways provide an estimate of the activation of defence mechanism in plants. Some of these enzymes are activated by the pathogenic attack, for example, LOX, PO, and PPO are activated during the response of plants upon pathogen ingress, called hypersensitive response (HR). Phenyl propanoid and oxylipin pathways also lead to production of antimicrobial compounds (3, 4).

Soybean (*Glycine max L.*, family Fabaceae), also known as "Golden bean", is a versatile crop having applications in food, feed, and other industries. Globally it contributes 25% in vegetable oil production. It also provides about two-thirds of the world's protein concentrate for livestock feeding and it is a valuable ingredient in formulated feeds for poultry and fish. Annually about 85% of the world's soybeans are processed into meal and oil. Soybean plants are affected by many diseases, such as seed diseases, seedling diseases, soybean cyst, sudden death syndrome, *Fusarium* root rot, brown spot, etc. (5). The pathogenic organisms include *Alterneria spp., Cercospora spp., Fusarium spp., Cladosporium, Phomopsis spp., Rhizoctonia spp., Pythium spp, Heterodera glycines,* and *Septoria glycines* (5, 6, 7). Bacteria earlier reported as PGPR for Soybean are *Azotobacter, Serratia, Azospirillum, Bacillus, Caulobacter, Chromobacterium, Agrobacterium, Erwinia, Flavobacterium, Arthrobacter, Micrococcus, Pseudomonas,* and *Burkholderia* (8).

Local Soybean varieties (grown in Gujarat region) are Gujarat soybean 1 & 2, JS – 335. The major pathogen causing seed and seedling diseases as well as root rot locally is *F. oxysporum*. In the present work, we have studied the efficacy of a PGPR (MP3) on growth and activation of the defense system in Soybean plants against *F. oxysporum*.

Materials and Methods

Fusarium oxysporum was procured from MTCC, Chandigarh, India. The Fungus was grown and maintained in Potato Dextrose agar medium. Isolate MP3, previously isolated from the rhizospheric soil of *Glycine max* L., was maintained in our lab on King's B agar medium. Soybean variety JS-335 was purchased from a local agro shop, Anand, Gujarat (India).

Effect of MP3 isolate on germinated seedlings of *Glycine max* L.

Germination assay was carried out by the method described earlier (9). Soybean seedlings were provided four treatments, A – control (No

treatments), B – treatment with bacterium isolate, MP3 C – challenged by *F. oxysporum,* and D – bacterium treated as well as fungal infected.

Three hundred seeds of soybean were washed in sterile distilled water three times followed by disinfection by 0.1% Mercury chloride for one minute. Seeds were again washed with sterile distilled water to remove the excess of mercury chloride on the surface of seeds and divided into two parts. One part (150 seeds) was treated with MP3 bacterial suspension containing 1×10^8 cells/ml and kept aside for two hours to coat with MP3 bacteria. The rest of the seeds, again parted into two. One (75 seeds) was imbibed in sterile distilled water which served as control and the rest (75 seeds) were infected with *F. oxysporum* spores (1×10^4 spores/ ml).

Half quantity (75 seeds) of dried seeds (MP3 coated) were transferred to a fresh beaker and added with fungal spore suspension to challenge them. Seeds were incubated for one hour followed by drying for another hour.

Five seeds of each treatment group were carefully placed using forceps into the petri plates over layered with damp sterile Whatman filter paper no.1 and incubated at 25 ± 2 ˚C temperature for 12 days. Plates were observed day to day and poured with the required amount of water. Sample of soybean seedlings from each treatment including zero-day was collected and stored at -20 ˚C for further analysis. On the twelfth day of germination root length, shoot length, fresh weight, dry weight, and percent germination were recorded. Vigor of seedlings was calculated using following equation.

Vigor = [Mean root length + Mean shoot length] x Germination Percentage

Effect of MP3 isolate on the growth of *Glycine max* L. plants

Seeds were treated in four different groups as explained above in germination assay. Twelve pots containing approximately 3 kg garden soil were sown with treated seeds by preparing a pit of 1 cm away from the surface and then covered with the soil. Water was sprinkled over it until seeds germinated to seedlings and then each pot was regularly watered sufficiently up to 35 days. When seeds turned to plants, root length, shoot length, fresh weight, dry weight, and number of leaves of each plant were recorded.

Defense-related enzyme studies.

Enzymes such as Phenyl ammonia lyase, Lipoxygenase, Peroxidase, Polyphenol oxidase, and Catalase were studied.

Extraction of enzyme.

Enzyme extraction buffer was prepared by following the method of Jiang and Zhang (9). The buffer contained 50mM sodium phosphate pH 7.0, 1 mM EDTA, 0.6% PVP and 1 mM PMSF. One gram of Soybean seedlings were crushed in a pre-chilled mortar pestle and added with 5 ml of the extraction buffer. The homogenate was centrifuged at 4 ˚C at 7000 rpm for 15 min. The supernatant was carefully transferred to fresh

Eppendorf tubes and stored at 4 ˚C for further analysis. Protein content was measured by Lowry's method to calculate the specific activity of enzymes.

Phenyl ammonia lyase (PAL) [EC 4.3.1.5]

The activity of PAL was measured by preparing a reagent mixture of 0.4 ml of enzyme extract, 0.5 ml of 0.1 M borate buffer mixed (pH 8.8) with 12 mM phenylalanine. It was allowed to incubate for 30 min at 30 ˚C. The product formed was measured by taking OD at 290 nm against a blank. Trans cinnamic formed as a product was calculated using extinction coefficient 9630 M^{-1} (10).

Lipoxygenase (LOX) [EC 1.13.11.12]

LOX activity was assayed using a reaction mixture containing 5µl of 25 mM substrate solution (linoleic acid), the 20 µl enzyme extract, and 2.975 ml of phosphate buffer pH 6.0 (10mM) solution to make up the final volume to 3ml. LOX activity was measured at an absorbance at 234 nm after one minute of incubation. Its specific activity was defined as µmol of conjugated diene Hydroperoxy-9,11-octadecadienoic acid (HPOD) produced per mg of protein per minute and calculated using a molar coefficient of 25000M^{-1} cm^{-1} (11).

Peroxidase (PO) [EC 1.11.1.7]

Peroxidase enzyme activity was measured following the method of Patel *et. al*, (2015). The substrate was prepared using 1.5 ml of 0.05 M pyrogallol, 0.5 ml of 0.1 M sodium phosphate buffer (pH 6.5). and 1% hydrogen peroxide. After addition of 0.5 ml of enzyme extract, change in OD was recorded every 30 seconds for 3 min. at 420 nm and was expressed ΔAbsorbance/ min/ mg of protein.

Polyphenol oxidase (PPO) [EC 1.14.18.1]

The reaction mixture for PPO activity consisted of 200 µL of the enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction, 200 µL of 0.01 M catechol was added and the change in O.D. was recorded at 30 s interval up to 3 min at 495 nm. The enzyme activity was expressed as changes in O.D. per minute at 495 nm (10).

Catalase (CAT) [EC 1.11.1.6]

Catalase activity was estimated by preparing 3 ml of the system. 800 µl of 200 mM of hydrogen peroxide was followed by 200 µl of crude enzyme in 100 mM potassium phosphate buffer. The reaction was measured at 240 nm (12).

Characterization of isolate MP3

Isolate MP3 was characterized by the morphological, biochemical, and molecular methods.

Morphological characteristics and Gram's Nature

Colony characteristics were observed and recorded. For Gram staining, the freshly grown bacterial colony was transferred to sterile distilled water to make a uniform suspension. A loop of

suspension was spread on a clear glass slide and allowed it to air dry. The slide was fixed by heating over a flame. Crystal violet solution was drained over a slide for 1 min followed by gram's iodine for 30 seconds. An excess amount of stain was poured off and the slide was gently washed under tap water. The slide was again washed with 90% alcohol to remove the unbound crystal violet - iodine complex from the cell wall of bacteria. Then the slide was covered with safranin, a counterstain, for 2 min. The stain was gently removed and the slide was washed with tap water and allowed it to dry. The slide was examined under a light microscope using 40X and 100X lenses. Violet color cells were identified as Gram-positive and pink color cells as Gram-negative. Observations were recorded.

Biochemical characterization of isolate MP3

Biochemical characteristics of the isolate were measured for its identification using a ready test obtained from Hi-media, Mumbai, India. The bacterial colony was point inoculated on a ready strip and was incubated for 24 hr. The results were recorded.

Molecular Characterization of MP3

DNA of isolate MP3 was extracted using a method described by Sambrook and Green (13). Isolated DNA was amplified and the PCR product was sent to 16s rRNA identification at Eurofines, Bengaluru, India. The obtained sequence was aligned with known bacterial sequences on NCBI using software MEGA7. The Gene sequence was submitted to Genebank.

Statistical analysis

Statistical analysis was done on a totally randomized sample using SPSS software version.22 (SPSS Inc.). The statistical analysis comprised of three replicates of each sample. The one-way ANOVA was carried out for the experiment. The significant difference was confirmed by Duncan's multiple range test (DMRT) at $P \leq 0.05$.

Results

Effect of MP3 isolate on the growth of *Glycine max* L seedlings

The effect of isolate MP3 on germination of *Glycine max* L. was studied by coating of freshly grown bacterial cell suspension on seeds of Soybean and observation of germinated seedlings. Percent germination of control, as well as *F. oxysporum* challenged seeds were increased from 46.66 % in control to 55.77% in MP3 coated seeds and 56.44 % in MP3 coated plus *F. oxysporum* challenged seeds (Table 1). MP3 improved the percent germination as well as all the vegetative parameters like root length, shoot length, fresh weight, dry weight, and vigor of Soybean seedlings (Fig 1). MP3 was found to increase root length, shoot length, fresh weight, and dry weight of seedlings significantly. It increased the vigor of soybean seedlings from 181.1 (control) to 302 (MP3) treated seedlings. Moreover, MP3 also significantly promoted the

vegetative growth and vigor of Soybean seedlings infected by *F. oxysporum.*

Fig.1 shows germination assay of Soybean seeds (JS-335 variety), where bacterial treated and bacterial treated + fungal infected seeds exhibit higher root length, shoot length.

Effect of MP3 on the growth of *Glycine max* L. plants:

The growth of bacterial treated plants was found better than the control and pathogenic fungus challenged and bacterial treated plants. It was observed that root length, shoot length, fresh weight, dry weight, and the number of leaves in Soybean plants increased significantly in the presence of MP3 isolate. It was also found to increase even in the presence of fungal pathogen *F. oxysporum.* Thus results of the pot assay corroborated the results observed in the germination assay.

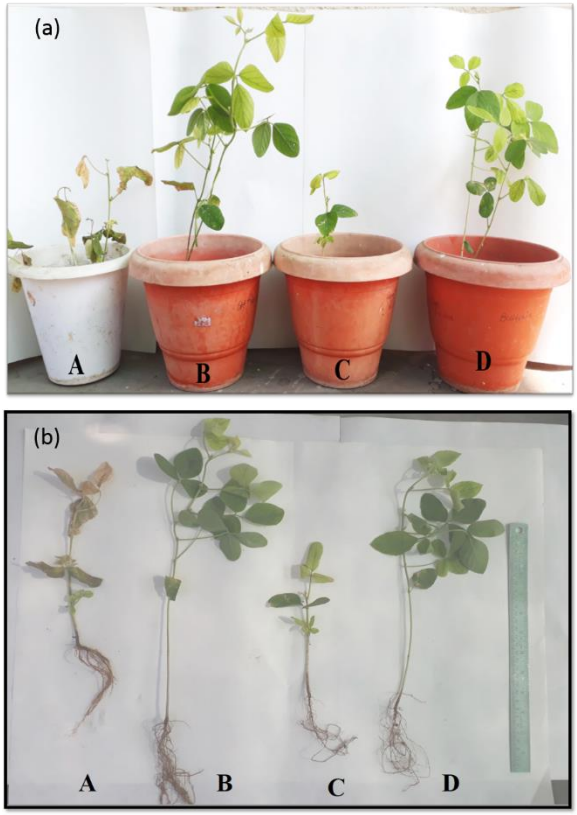

Fig. 2 Pot assay of *Glycine max* L (JS-335). (a) Differential growth of differently treated plants (b) Plants showing vegetative growth, where, A – control, B – MP3 treated, C – Fungal infected (*F.oxysporum*), D – MP3 treated+ fungal infected (*F.oxysporum*).

Effect of MP3 on the induction of defense-related enzymes

PAL activity

Phenylalanine ammonia lyase (PAL), the key enzyme in the phenylpropanoid pathway, was assayed from control and treated germinated seedlings. In fungal infected seedlings, PAL activity was found lower than control, indicating lack of phenol synthesis and defence response.

	Germination%	Root Length	Shoot Length	Fresh Weight	Dry Weight	Vigor
Control	46.66 ± 15.27^{ab}	2.80 ± 0.74^{cd}	0.93 ± 1.20^{a}	0.344 ± 0.14^{ab}	0.122 ± 0.11^{a}	181.12
MP3	55.77 ± 18.85^{ab}	$3.71 \pm .14^{abcd}$	1.51 ± 0.83^{a}	0.404 ± 0.14^{ab}	0.149 ± 0.07^{a}	302
Fungus	36.66 ± 18.55^{b}	2.21 ± 1.17^{d}	1.07 ± 1.63^{a}	0.286 ± 0.14^{b}	0.081 ± 0.06^{a}	132.67
MP3 + F	56.44 ± 14.86^{ab}	$3.43 \pm .40^{abcd}$	1.74 ± 1.81^{a}	0.341 ± 0.17^{ab}	0.120 ± 0.05^{a}	312.69

(Significance at $p \geq 0.005$, Duncan's DMRT test)

Table 1 Vegetative parameters of twelve-days-grown germination seedlings. It indicates that MP3 treated seedlings significantly enhanced the overall growth of soybeans.

	Root Length	Shoot Length	Fresh Weight	Dry Weight	Number of leaves
Control	13.49 ± 1.74^{ef}	6.70 ± 1.28^{ab}	1.638 ± 0.14^{de}	0.535 ± 0.02^{fg}	16.77 ± 1.07^{f}
MP3	19.33 ± 0.90^{bc}	8.53 ± 2.03^{ab}	1.760 ± 0.07^{cd}	0.909 ± 0.08^{d}	18.94 ± 1.29^{ef}
Fungus	11.07 ± 1.97^{f}	4.40 ± 1.19^{b}	1.082 ± 0.12^{f}	0.453 ± 0.04^{g}	12.11 ± 1.83^{g}
MP3 + F	18.40 ± 2.82^{bc}	10.41 ± 4.67^{ab}	1.976 ± 0.47^{bc}	0.929 ± 0.08^{d}	24.36 ± 1.76^{bc}

(Significance at $p \geq 0.005$, Duncan's DMRT test)

Table 2 Pot assay results of vegetative growth parameters of 35-days-grown plants. Root and shoot length, fresh weight, dry weight, and number of leaves of Soybean plants treated with MP3 bacterial isolate were significantly higher than the control. All these parameters were also increased even in the presence of fungal pathogen *F.oxysporum*

PAL activity was increased in MP3 treated uninfected as well as infected seedlings, indicating elicitation of plant defence. In MP3 treated seedlings increase in PAL activity was observed much earlier, but in fungal infected MP3 treated seedlings, it increased from the fourth day onwards, showing significance of MP3 in early mounting of resistance. Higher PAL activity was maintained further as measured up to eight days.

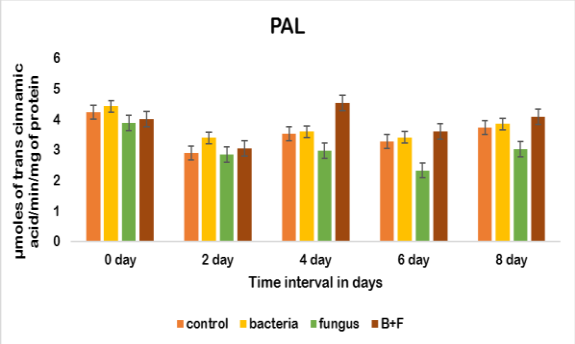

Fig. 3 PAL activity was estimated from germinated seedlings of soybeans grown for different number of days. Infected as well as uninfected seedlings treated with MP3 bacterial isolate showed higher PAL activity. Error bar indicates SD value.

LOX Activity

LOX activity was found to be elevated in combined treatment of MP3 and *F. oxysporum* in Soybean seedlings rapidly, but sustained only for two days, whereas only MP3 treatment showed delayed and lesser induction of the LOX activity except on the fourth day.

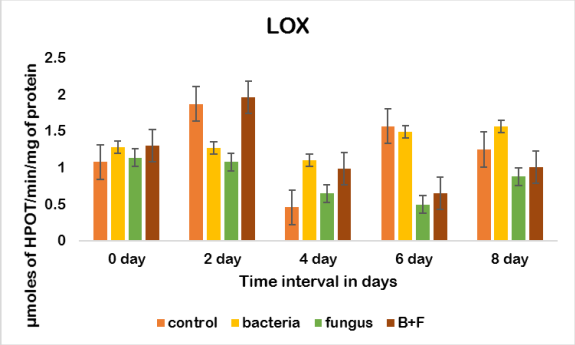

Fig.4 LOX activity from different-days-old Soybean seedlings. Treatment of MP3 isolate induced LOX activity from the fourth day, however, combined treatment of MP3 isolate and *F. oxysporum* showed higher LOX activity from day 2.

PO Activity

A significant increase in peroxidase activity in MP3 treated uninfected as well as infected Soybean seedlings were observed, indicating the role of MP3 in the induction of systemic resistance in *G. max* L. In seedlings infected with *F. oxysporum* only (not treated with the isolate MP3), there was no increase in peroxidase (Fig 5).

PPO Activity

A significant increase in PPO activity was found due to MP3 bacterial treatment in both fungal infected and uninfected Soybean seedlings, although infection of *F. oxysporum* also induced PPO activity to some extent (Fig 6). The increase in PPO activity supports the observation that MP3 mounts ISR in *G. max* L. seedlings and is backed up by similar observations in ISR generated in other plants (14, 15).

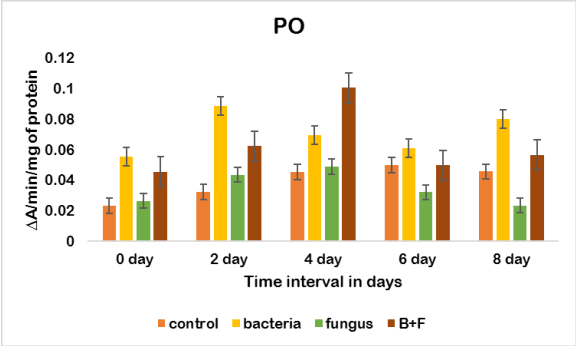

Fig.5 Activity of PO enzyme from different-days-old Soybean seedlings. Bacterial isolate MP3 induced the PO activity in both uninfected as well as infected plants. Bars in sequence: Control, Bacteria (MP3) treated, fungus infected and Bacteria treated as well as fungal infected (B+F).

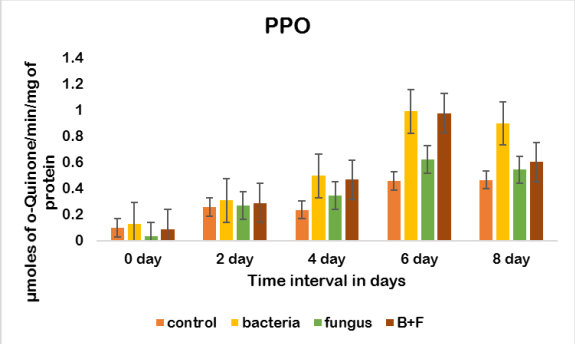

Fig.6 Activity of PPO enzyme from different-days-old Soybean seedlings. Activity of PPO was found to increase rapidly due to the treatment of PGPR MP3.

Catalase Activity

The activity of catalase was found to be different in comparison to controls on different days, probably due to varied amount of reactive species in plant tissues.

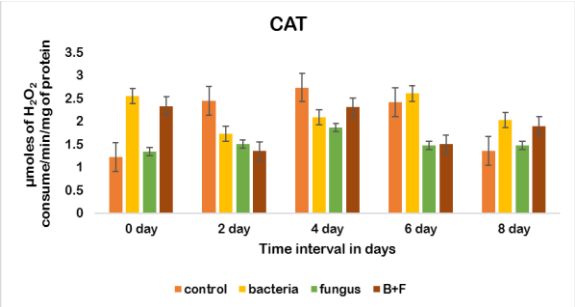

Fig.7 Activity of Catalase enzyme from different-days-old Soybean seedlings. Catalase was differentially induced in MP3 bacteria treated seedlings.

Identification of Isolate MP3

Morphological characteristics and Gram's Nature of MP3 Isolate.

Morphological characteristics of the freshly grown isolated colony of MP3 on King's B agar medium were observed and the observations were recorded (Table 3). Isolate MP3 was found to be Gram-negative tiny rod-shaped bacteria (Fig 8).

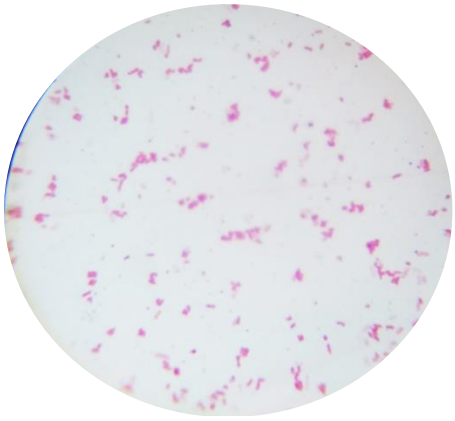

Fig.8 Gram staining of MP3

Biochemical characteristics of MP3 Isolate.

Isolate MP3 was found positive for Voges Proskauer's, Citrate, ONPG, Nitrate Reduction, Catalase, Arginase, Glucose, Arabinose, Trehalose, Mannitol, Sorbitol, and Sucrose. Biochemical characteristics were found to match with bacterial genera *Serratia*.

Molecular characterization of MP3 Isolate.

Genomic DNA was isolated from MP3 isolate and the 16s rRNA partial gene sequence was amplified by PCR (Fig 9 a & b). Sequencing and alignment of the 16s r RNA gene using BLAST on NCBI showed 99% similarity with *S. marcensecns*. The Gene sequence was submitted to Genebank and the accession number obtained was MK073018. The phylogenetic neighbour joining tree was prepared using MEGA7 software. Bacterial 16s rRNA gene sequence was found to be closely related to *S. marcensecns* with high bootstrap value (Fig 10). The tree was prepared by a bootstrap bar of 0.005 basepairs.

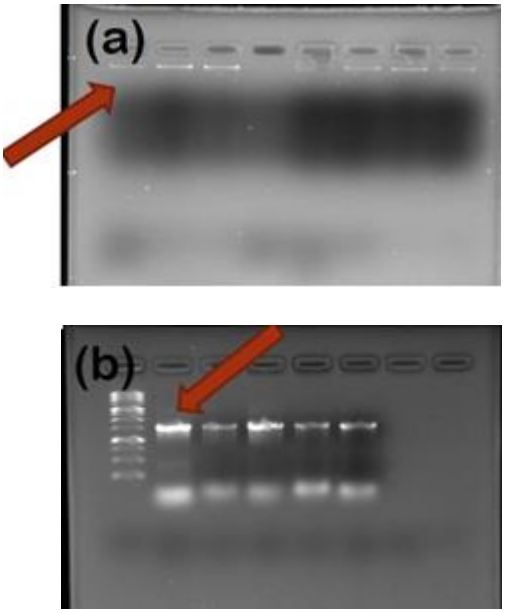

Fig.9 (a) DNA band on 0.8 % Agarose gel. (b) PCR band of 1500 bp on 0.8 % Agarose gel.

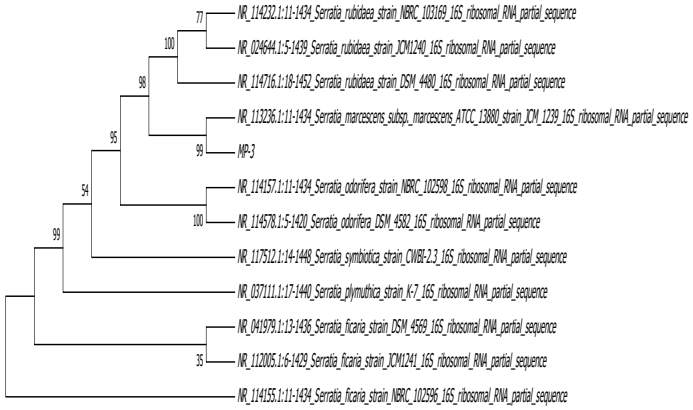

Fig 10 Position of MP3 bacterial isolate in a neighbour-joining tree.

Discussion

In the present study, we have characterized plant growth-promoting characteristics of one of the isolates, MP3 isolated from the rhizosphere of *Glycine max* L. plants. MP3, identified as *S. marcescens* improved the germination of Soybean seedlings, promoted the growth of plants, and controlled one of the most important pathogens of Soybean, *Fusarium oxysporum*. It was observed that seedlings and plants infected with *F. oxysporum* neither grew well nor showed induction of systemic resistance, whereas if co-treated with *S. marcescens*, they showed improved growth and better mounting of ISR. *S. marcescens* is earlier reported to promote the growth of coconut plants (8).

Synthesis and accumulation of phenols by phenylpropanoid pathway is the primary

inducible defense response in plants against a number of biotic and abiotic stresses leading to the synthesis of defense metabolites like phytoalexins, coumarins, flavonoids, and precursors of lignin and suberin (12, 15, 16,). The activity of the first enzyme of this pathway, PAL was induced by *S. marcescens,* which is one of the factors for the mounting of resistance and uninhibited growth of *F. oxysporum* infected Soybean plants (Figure 2). An increase in PAL activity due to the induction of systemic resistance in *Glycine max* L. infected with *F. oxysporum* has been reported earlier (15, 16). Lipoxygenase enzyme, responsible for lipid peroxidation, loss of membrane integrity, and electrolyte leakage is induced in a number of plant-pathogen interactions (4). LOX activity was not induced upon treatment of this bacterium, however it did increase upon combined treatment with *F. oxysporum*. These results are corroborated with many reports showing that LOX is generally activated by the necrotrophic pathogen. *F. oxysporum* being a hemibiotroph (initially biotrophic but later necrotrophic) was able to activate LOX (17). Activities of PO and PPO were induced in bacterium treated seedlings. An increase in PO and PPO activity as a part of ISR has been reported in many plants infested with *F. oxysporum* (14, 15). Catalase activity was found varying on different days. Catalase along with other antioxidant enzymes clears ROS in plant tissues infected with pathogens (generated during HR). As catalase transforms reactive hydrogen peroxide into water, its activity depends upon the amount of ROS levels in plant cells and different plant tissues express different catalase activity in response to *Fusarium* infection (10, 12, 15).

Tests of Biochemical characteristics of the isolate identified that it belongs to genus *Serratia,* based on positive results for Voges Proskauer's, Citrate, ONPG, Nitrate Reduction, Catalase, Arginase, Glucose, Arabinose, Trehalose, Mannitol, Sorbitol, and Sucrose, as well as literature support (18). Based on this as well as on the alignment of the 16s rRNA gene with bacterial sequences on NCBI, this isolate was identified as *Serratia marcescens. S. marcescens* has been previously reported as PGPR and showed similar biochemical characteristics (18), however, this is the first report of *S. marcescens* promoting the growth of *Glycine max* L. plants.

Conclusion

A PGPR isolate MP3 increases the germination, promotes growth, and induces

the defense mechanism against *F. oxysporum* in *Glycine max* L. plants. Using biochemical and molecular characterization, it was identified as *S. marcescens*. Based on the present studies it can be concluded that *S. marcescens* can be used as a PGPR in Soybean plants and as a biocontrol agent against *F. oxysporum* after confirmation of its performance at the field level.

Acknowledgement

Authors are thankful to Post Graduate Department of Biosciences, Sardar Patel University for providing infrastructure and all necessary facilities.

Funding information

No specific funding was received to conduct the study.

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Bacteria Name	**Size**	**Shape**	**Edge**	**Elevation**	**Surface**	**Consistency**	**Pigment**
Mp3	Small	Round	Entire	Colonial	Alveoli	Moist	Orange, Red to Maroon

Table 3 MP3 Morphological characteristics

PRAJÑĀ - **Journal of Pure and Applied Sciences Vol. 27-28: 26 - 40 (2019-20)**
ISSN 0975 - 2595

Study of biosynthesis and statistical optimization of medium components for β-carotene production using marine *Paracoccus* sp. OC1 isolated from Gulf of Khambhat

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ABSTRACT

The present study aimed to isolate and identify a carotenoid producing microbial strain from the marine environment. The orange-colored microbial colony was isolated from marine water of the Gulf of Khambhat, Gujarat, India, and identified as a Paracoccus species using 16s rRNA gene amplification. Environmental factors viz. medium strength, pH, temperature, sugar, and aeration affecting the carotenoid production were optimized. Paracoccus spp. OC1 produced the highest pigment in media-Marine broth (MB) 1/10, pH-7, temperature-27ºC, sugar- sucrose, with incubation at static condition. Besides the five-fold increase in β-carotene production was achieved by optimization of meat extract, glycerol, ferric citrate, and potassium nitrate concentration using statistical designs like Plackett-Burman design and response surface methodology. The β-carotene was purified using thin layer chromatography and characterized using UV-Visible spectroscopy, FTIR spectroscopy, and mass spectroscopy. The presence of the lycopene β-cyclase gene responsible for β-carotene production was determined through PCR amplification.

***Keywords:** Carotenoids, Plackett-Burman Design, RSM, FT-IR, MS.*

INTRODUCTION

Natural products with diverse structures and applications gain importance in pharmaceutical, nutraceuticals, and food industries over synthetic products. One such product category is pigments of natural origin that include chlorophyll, melanins, carotenoids, etc. Carotenoids with a wide color range from yellow to red, present in plants, algae, fungi, and bacteria, are very attractive and widely used as a potential antioxidant in nutraceuticals. Carotenoids serve as a source of vitamin A (α-carotene, β-carotene, and β-cryptoxanthin), increasing the functioning of the immune system, preventing the cell from damage due to their antioxidant effect [1, 2]. This property is useful for preventing certain types of cancers in humans by serving as a hormone precursor, in photoprotection and increasing the growth rate of cells [3, 4]. Carotenoids play a major role in quenching singlet oxygen radicals and removing molecular oxygen from a biological system without producing any oxidizing products. Moreover, β-carotene traps peroxy free radicals in tissues at a low oxygen concentration [5, 6]. The growth of commercially used carotenoids increases at the rate of 2.3% annually with nearly $ 1.4 billion estimated market value in 2018. Specifically, the market value of β-carotene is expected to grow $334 million by 2018 with a 3.1% annual growth rate [6]. Large numbers of bioactive compounds bearing therapeutics applications were obtained from marine sources, as they have a highly beneficial effect on human health. Peng et al. [7] demonstrated the absorbance of carotenoids and their derivatives (e.g. fucoxanthin and fucoxanthinol) in the digestive tract of mammals which could enter in blood circulation system. Marine carotenoids like fucoxanthin and its metabolites fucoxanthinol, amarouciaxanthin A, and halocynthiaxanthin have antioxidant, anti-inflammatory, anticancer, anti-obese, antidiabetic effect and also have protective

effects on the liver, blood vessels of the brain, bones, skin, and eyes [8]. Therefore, the Gulf of Khambhat, a saline-alkaline site, was explored to obtain a pigment-producing microorganism. Depending upon the physicochemical factors viz., medium components, pH of media, temperature, aeration, presence of inorganic salts, and organic carbon source of the medium, the carotenoids producing ability of bacteria differ [9]. With excellent market value and applications of carotenoids, especially β-carotene, there is a need for scaling up the production using microbial source. Hence, the present study aimed to optimize parameters for carotenoids production, followed by developing medium formulation using statistical approach for increased carotenoid production by *Paracoccus* sp. OC1 isolated from Gulf of Khambhat. Carotenoid produced was separated and characterized using chromatographic and spectroscopic methods. The Lycopene β-cyclase, a key enzyme responsible for β-carotene production, was amplified from genome of *Paracoccus* sp. for the confirmation of β-carotene synthesis.

METHODOLOGY

Microorganism: Isolation and Identification

The water sample was collected from the Gulf of Khambhat (22.3°N 72.62°E) in a sterile bottle and stored at room temperature until used. The water sample was serially diluted and inoculated on various media like marine agar, Luria agar, and nutrient agar (Himedia, Mumbai, India), using the spread plate technique and incubated at 27°C for 4-5 days till colored colonies appeared for isolation of desired pigmented microorganism. The orange color colony was selected and maintained on marine agar by routine subculturing [10]. For the identification of bacteria, biochemical tests were performed and the 16S rRNA gene was amplified using universal forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer (5'-AAGGAGGTGATCCAGCCGCA-3') purchased from 1st BASE (Agile Life Science Technologies India Pvt. Ltd.). Amplified Gene product was sequenced at 1st BASE (Agile Life Science Technologies India Pvt. Ltd.). Using the Nucleotide BLAST search program [11] (http://www.ncbi.nlm.nih.gov/blastn) nucleotide sequence homology was searched and a neighbor-joining (NJ) tree with bootstrap value 500 was generated using the MEGA 4.0 software [12]. The sequence was submitted to GenBank [13].

Optimization of preliminary growth parameters and total carotenoids (TC) estimation

For studying primary growth parameters, 1% of actively growing culture was inoculated in different media and all flasks were incubated at 27°C for 7 days. Fermentation parameters like medium strengths (MB, MB 1/2, and MB 1/10), pH of media (pH 5, 7, 9, 10, 11, and 12), incubation temperatures (27°C, 37°C and 45°C) and carbon source (starch, sucrose, glucose, maltose, and glycerol; 1% of each sugar) were used for optimizing growth and pigment production. The effect of agitation was studied by incubating flasks on static (0 rpm) and shaking (150 rpm) conditions at 27°C. The intracellular pigments were extracted by the cell lysis method. For the determination of total carotenoids (TC) content of a cell, 1 mL of culture was centrifuged and the weight of the cell pellet was determined by the gravimetric method [14]. The pigment was extracted from the cell pellet using different solvents like methanol, acetone, chloroform, hexane, and ethyl acetate at 4°C until the cell pellet becomes colorless. The cell debris removed by centrifugation and supernatant with crude carotenoids was used for further analysis. All the tubes were kept in dark to prevent the oxidation of carotenoids [15]. Separation and characterization of carotenoids were done using chromatographic technique and spectroscopic analysis.

Identification of a gene involved in β-carotene biosynthesis

Under the present study, three enzymes lycopene β-cyclase, β-carotene hydrolase, and β-carotene ketolase playing role in carotenoid biosynthesis were amplified from the genomic DNA of *Paracoccus*. To identify these three enzyme, primer used were FP_LBC 5'-gcttgaggtgtcgctggt-3' and RP_LBC 5'-gtggaaaaaccggtgaaaga-3' for lycopene β-cyclase; FP_BCH 5'-gactgctgctgcgactcc-3' and RP_BCH

5'-gctccaaaactcccctctct-3' for β-carotene hydrolase and FP_BCK 5'-catcccctcatcttccttca-3' and RP_BCK 5'-caccaggccaggtaaggata-3'for β-carotene ketolase. The reaction was carried out in a 50 µl reaction mixture containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 25 pmol of Forward Primer (FP), and Reverse Primer (RP) of a particular enzyme, 50 ng DNA template, and 5 U Taq DNA polymerase. Amplification cycle parameters were set as 94 ˚C for 1 min for initial denaturation then 94 ˚C for 45 sec, 60 ˚C for 45 sec and 72 ˚C for 60 sec, for 35 cycles followed by a final extension of 10 min at 72 ˚C in a thermocycler. Amplified PCR products were analyzed using agarose gel electrophoresis (1.5 %) and compared with a 100 bp DNA ladder to determine the product size.

Screening of carbon and nitrogen source using a statistical approach

Plackett-Burman Design

The Plackett–Burman design, an effective technique for screening of 11 medium components [16–18], was used concerning their main effects on total carotenoids production. Based on our preliminary studies using One-Variable-At-a-Time (OVAT), the carbon and nitrogen sources were further used to show their main effect on TC production included in the design [19].

The effect of each variable was determined by equation (1):

$$E_{(xi)} = (\Sigma\ TCi^{+} - TCi^{-}) / N \text{----------(1)}$$

Where $E_{(xi)}$ is the concentration effect of the tested variable. TCi^{+} and TCi^{-} are the total carotenoids from the trials where the variable (*x*i) measured were present at high and low concentrations, respectively; and N is the number of trials divided by two.

Total eleven factors, glucose, starch, sucrose, glycerol, maltose, fructose, ferric citrate, meat extract, yeast extract, potassium nitrate, and ammonium nitrate were screened for their effect on the final product. The salinity was set up to 3.5% with artificial marine water in each run [20]. In the present study, a total of eleven components were screened using 18 experimental runs with six center point. TC production was carried out in triplicate and the average value was taken as the response. The factors showed a significant positive effect at 95% level ($P < 0.05$) were considered to have a significant effect on TC production and thus used for further optimization to formulate the final medium by response surface methodology (RSM).

Response Surface Methodology

Total four variables showing the highest positive effect in Plackett-Burman design were further used to formulate final media based on variable screened previously using central composite design (CCD) with a five-coded level. For four variables this design was made up to full 2^4 factorial design with the four cube points and total run number for CCD concerning the concentration of components could be decided by full factorial points $2^k$where *k* is the number of variables, center points η0 (η0>1) (six replicates) and two axial points for each variable (á=2^k/4, =2 for k=4) total design points (experiments) would be N=2^k+2^k+ η0=30 [21, 22].

The second-order polynomial coefficients were calculated and analyzed using the ''Design Expert" software (Version 9.0.3.1, Stat-Ease Inc., Minneapolis, USA) statistical package. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). For the informative analysis of variables, linear, mean, quadratic, quartic, and cubic terms of coded levels of glycerol, potassium nitrate, meat extract, and ferric citrate were tested. The level at which every term in the selected model should be significant was set at 5% [19]. The response surface plots were generated by the same software and response was calculated by using the following equation (2):

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_1\beta_2 AB + \beta_1\beta_3 AC + \beta_1\beta_4 AD + \beta_2\beta_3 BC + \beta_2\beta_4 BD + \beta_3\beta_4 CD + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 \text{----------(2)}$$

where Y is the response in terms of TC production, β_0 is the model intercept and β_1, β_2, β_3, β_4 are linear coefficient, β_{11}, β_{22}, β_{33}, β_{44} are squared coefficient and $\beta_1\beta_2$, $\beta_1\beta_3$, $\beta_1\beta_4$, $\beta_2\beta_3$, $\beta_2\beta_4$, $\beta_3\beta_4$ are interaction coefficient and A, B, C, D, AB, AC, AD, BC, BD, CD, A^2, B^2, C^2, and D^2 are level of the independent variable [23].

RESULTS

Isolation and Identification

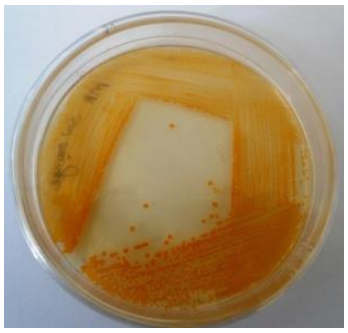

Fig. 1. **Growth of *Paracoccus* spp. on marine agar plate having orange pigmentation.**

The color of the colony was the basis of primary screening for the isolation of a carotenoid producing strain, therefore an orange-colored colony was isolated and maintained on a marine agar plate (Fig. 1) and labeled as OC1. Colonies were small, round with entire margin and convex elevation which showed a smooth surface with moist consistency.

Table : Result of Biochemical tests for *Paracoccus* spp., OC1

Biochemical Tests	Reaction
Gram Reaction	-
Spore Formation	-
Methyl Red Test	-
Voges- Proskauer (V-P) Test	-
Citrate Utilization Test	-
Indole Production Test	-
Hydrogen Sulphide Production Test	-
Phenylalanine Determination Test	-
Nitrate Reduction Test	-
Gelatine Hydrolysis Test	-
Ammonia Production Test	-
Urea Hydrolysis Test	-
Starch Hydrolysis Test	-
Casein Hydrolysis Test	-
Catalase Test	+
Dehydrogenase Test	-
Triple Sugar Iron Agar Test	-
Carbohydrate Utilization Test	
Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Trehalose, Malibiose, Sucrose, L-Arabinose, Mannose, Inulin, Glycerol, Inositol, Sorbitol, Adonitol, Arabitol, Erythritol, Cellobiose,Xylitol, D-Arabinose	+
Raffinose, Sodium Gluconate, Salicin, Dulcitol, Mannitol, α-methyl D-glucoside, Rhamnose, Melezitose, α-methyl D-mannoside, ONPG, Esculin Hydrolysis, Malonate Utilization, Sorbose	-

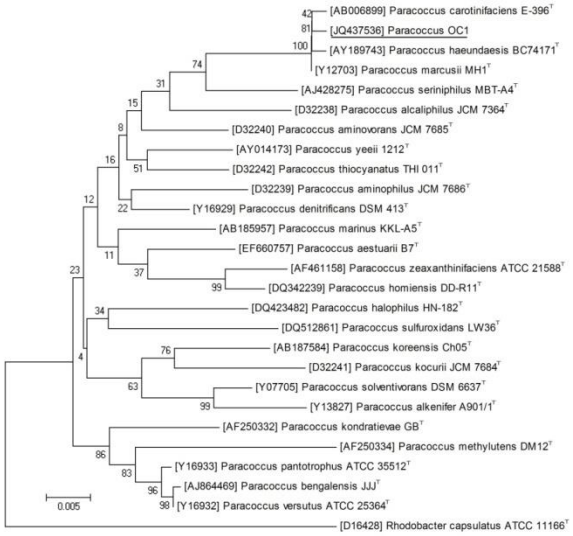

Fig. 2. **Phylogenetic neighbor-joining tree based on 16S rRNA gene sequences showing the isolates having closely relationship with *Paracoccus carotinifaciens* E-396 ^{T} and bootstrap values (expressed as percentages of 500 replications) greater than 50% are given at nodes. Bar 0.5% sequence variation.**

Microscopic observations suggested that strain OC1 was Gram's negative, nonmotile, rod-shaped, and nonspore forming. The biochemical characteristics of the OC1 strain are represented in Table 1. A comparison of the morphological and biochemical traits of OC1 with *Paracoccus* spp. [24] suggested that OC1 belonged to *Paracoccus* genus. For further identification of the strain, phylogenetic analysis was performed by aligning the 16S rRNA gene sequence of type strains reported till date belonging to *Paracoccus* genus with that of OC1 to construct a phylogenetic tree. Based on the phylogenetic tree, the type strain *P. carotinifaciens* E-396^{T} [AB006899] was the closest neighbor of *Paracoccus* OC1 with 99.35% similarity (Fig. 2). The 16s rRNA gene sequence was submitted GenBank nucleotide sequence database under the accession number JQ437536. Besides, *P. carotinifaciens* E-396^{T} was reported to produce astaxanthin [25] but our isolated strain can produce β-carotene, which is a different type of carotenoid, suggesting *Paracoccus* OC1 to be considered as a novel strain.

Preliminary Physiological Growth Parameter Optimization and Total Carotenoids Estimation

For biomass and carotenoid production, different physiological parameters were studied using OVAT method.

Table 2: Effect of preliminary growth parameter like medium strength, pH, incubation temperature, carbon source and aeration on wet cell weight and TC production.

	Wet Cell Weight (gm/L)	Total Carotenoids Weight (mg/L)
Media		
MB	28.33 ± 1.15	833.33 ± 288.67
MB1/2	20.33 ± 2.56	666.66 ± 188.67
MB1/10	14.5 ± 2.59	566 ± 148.58
pH of media		
pH 7	17.16 ± 0.28	565.33 ± 36.89
pH 8	21.16 ± 2.56	932.66 ± 73.54
pH 9	18 ± 1.32	867.33 ± 55.14
pH 10	17.66 ± 1.75	752 ± 102.05
Incubation Temperature		
27°C	28.33 ± 1.15	833.33 ± 288.67
37°C	17.66 ± 2.75	1166.66 ± 288.65
47°C	14.83 ± 6.71	545 ± 38.25
1% of each Sugar in medium MB		
Starch	22.5 ± 2.64	833.33 ± 288.67
Sucrose	18.33 ± 2.02	1166.66 ± 267.88
Glucose	19.16 ± 1.89	1090 ± 108.58
Maltose	20 ± 2	1000 ± 58.65
Glycerol	25.66 ± 3.25	1333.33 ± 577.35
Aeration		
Static Condition	28.33 ± 1.15	833.33 ± 187.36
Shaking Condition	16.5 ± 0.86	500 ± 87.36

Table 2 shows results of physico-chemical optimization for biomass and total carotenoids production. The biomass production was 26.16 g/L, 19.0 g/L, 12.83 g/L and corresponding carotenoid production was 833.33 mg/L, 666.66 mg/L, 500 mg/L in three different medium concentrations MB, MB (1/2), and MB (1/10) respectively. Microorganisms grew in the pH range of 7 to10, at pH-8, the highest biomass 21.16 g/L, as well as carotenoid production of 932.66 mg/L, was observed, whereas media having pH-5, 6, 11, and 12 were not conducive for the growth of *Paracoccus* sp. OC1. Isolate OC1 was growing at a temperature range of 27°C to 45°C and produced the highest biomass 28.33 g/L at 27°C and highest carotenoid 1166.67 mg/L when incubated at 37°C. Among five different sugars, glycerol supports the highest biomass (23.5 g/L) and total carotenoid (1333.33 mg/L) when incubated at static conditions.

Separation and characterization of β-carotene

Extracted carotenoid was analyzed by chromatographic and spectroscopic techniques. Concentrated methanolic extract of carotenoids was loaded on a TLC sheet using a glass capillary along with standard β-carotene (Hi-media) and developed using methanol: ethyl acetate (1:1 v/v) solvent system. The retention factor (R_f) of separated pigment was similar to standard β-carotene. The colored band separated corresponding to standard β-carotene was scraped and dissolved in methanol and used as a sample for spectroscopic analysis. In UV-Visible spectroscopy, three peaks at 425 nm, 450 nm, and 475 nm were obtained, which shows the signature spectral pattern of carotenoids [19, 26]. In Fourier Transformed Infra-Red (FT-IR) Spectroscopy, the spectrum exhibited peaks at 3436.54 cm^{-1} and 2923.50 cm^{-1} for asymmetric and symmetric stretching vibration of $-CH_2$ and $-CH_3$, 1633.77 cm^{-1} for a stretch of -C=C- (alkenes) repeats more than four to five times, 1028.78 cm^{-1} for trans conjugated alkenes -CH=CH- out of plane deformation mode which represents the carotenoid have similarity with the functional groups present in the structure of β-carotene [27]. Mass spectra of purified pigment showed a 100% mass to charge ratio at 538.25 (M^+H). Breemen reported molecular ion of β-carotene showed a signal of mass to charge ratio (m/z) at 536 (M) [28]. In another study, the mass spectrum of β-carotene predominance molecular ion (M^+H) showed m/z ratio at 537.33 [29]. From all the analytical techniques we proposed that the extracted molecule was β-carotene.

Amplification of gene involved in β-carotene biosynthesis

Out of all enzymes responsible for carotenoid biosynthesis, only three enzymes; lycopene β-cyclase, β-carotene hydrolase, and β-carotene ketolase were selected and their respected gene sequences were searched using KEGG (Kyoto Encyclopedia of Genes and Genomes) database [30]. Expected gene product size of lycopene β-cyclase, β-carotene hydrolase, and β-carotene

ketolase were 1050 bp, 918 bp, and 1599 bp respectively [31–33]. Figure 3 shows agarose gel containing PCR amplified products of selected genes with 100 bp DNA ladder and genomic DNA of isolate OC1. In PCR amplification, only lycopene β-cyclase gene was amplified with product size ~950 bp, and the expected gene size was 1050 bp, which showed the presence of the gene in genomic DNA of *Paracoccus spp*. OC1.

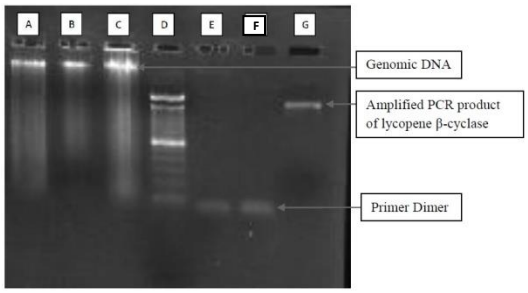

Fig. 3. Agarose gel electrophoresis of genomic DNA isolated from *Paracoccus* OC1 (A, B, C), 100bp DNA ladder (D), amplified PCR products for lycopene β-cyclase (G), β-carotene hydrolase (F) and β-carotene ketolase (E) in which only well G contain amplified gene where as well E and F showed only primer dimer, no amplification of gene.

Statistical Design

Plackett-Burman Design

Primary screening of physiological parameters and sugar utilization was performed by the OVAT method. Based on these results, eleven components were screened using the Plackett-Burman design.

Table 3: Effect of components used in Plackett-Burman design with their standardized effect and % contribution on TC production.

Variable	+ value (gm/L)	- value (gm/L)	Standardized effect	% Contribution
X1 (Glucose)	0.5	0.05	-83.33	3.05
X2 (Starch)	0.5	0.05	-59.33	1.55
X3 (Sucrose)	0.5	0.05	-208.67	19.12
X4 (Glycerol)	0.5	0.05	266.00	31.07
X5 (Maltose)	0.5	0.05	-22.00	0.21
X6 (Fructose)	0.5	0.05	-3.33	4.879E-003
X7 (Ferric citrate)	0.1	0.01	3.33	4.879E-003
X8 (Meat Extract)	2	0.2	90.00	3.56
X9 (Yeast Extract)	1	0.1	-106.00	4.93
X10 (Potassium Nitrate)	0.5	0.05	110.00	5.31
X11 (Ammonium Nitrate)	0.5	0.05	146.00	9.36

Table 3 shows the concentration of each parameter with its standardized effect on TC with a % contribution. Of the eleven tested components, glycerol had a maximum positive effect on TC production, followed by ferric citrate, meat extract, ammonium nitrate, and potassium nitrate. Table 4 shows the design matrix of each nutrient component tested at two concentrations, high and low concerning TC production ranging from 136 mg/L to 896 mg/L.

Table 4: Plackett-Burman design for eleven factors with two coded level (+ and -) with their respective TC production in each run.

Run	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	TC (mg/L)
1	+	+	-	+	+	+	-	-	-	+	-	688
2	-	+	+	-	+	+	+	-	-	-	+	366
3	+	-	+	+	-	+	+	+	-	-	-	544
4	-	+	-	+	+	-	+	+	+	-	-	652
5	-	-	+	-	+	+	-	+	+	+	-	340
6	-	-	-	+	-	+	+	-	+	+	+	896
7	+	-	-	-	+	-	+	+	-	+	+	724
8	+	+	-	-	-	+	-	+	+	-	+	464
9	+	+	+	-	-	-	+	-	+	+	-	136
10	-	+	+	+	-	-	-	+	-	+	+	824
11	+	-	+	+	+	-	-	-	+	-	+	472
12	-	-	-	-	-	-	-	-	-	-	-	480
13	0	0	0	0	0	0	0	0	0	0	0	532
14	0	0	0	0	0	0	0	0	0	0	0	652
15	0	0	0	0	0	0	0	0	0	0	0	792
16	0	0	0	0	0	0	0	0	0	0	0	852
17	0	0	0	0	0	0	0	0	0	0	0	652
18	0	0	0	0	0	0	0	0	0	0	0	668

Response Surface Methodology

The four components with the highest positive effect on TC production were used to further optimize and develop models using the central composite design. Table 5 shows the production of TC ranging from 1156 mg/L to 5884 mg/L when changing the different concentrations of the components.

Model development

Selection of sequential model by the sum of square

Appropriate selection of the model to calculate and estimate the response is of key importance. The quadratic model provided a significant value of P 0.0002 (<0.05) with fourteen degrees of freedom. Therefore, it was chosen to analyze the effect of the variables in terms of the main effect, the interactive and quadratic effects. The variance analysis (ANOVA) for the quadratic model was calculated from the response obtained for 30 runs.

Table 5: RSM design of four components at five concentration levels and their respective TC production (A=Glycerol, B=Potassium Nitrate, C=Meat Extract, and D=Ferric Nitrate with central value of 0.75, 0.75, 3.5 and 0.075 g/L concentration as a central value with six replicates).

Run	A (g/L)	B (g/L)	C (g/L)	D (g/L)	TC (mg/L)	
					Actual	Predicted
1	0.5	0.5	2.0	0.05	2548	2441.83
2	1.0	0.5	2.0	0.05	2800	2845.33
3	0.5	1.0	2.0	0.05	2952	2405.33
4	1.0	1.0	2.0	0.05	3008	2531.83
5	0.5	0.5	5.0	0.05	4484	4646
6	**1.0**	**0.5**	**5.0**	**0.05**	**5884**	**5404.5**
7	0.5	1.0	5.0	0.05	4676	4500.5
8	1.0	1.0	5.0	0.05	4660	4982.0
9	0.5	0.5	2.0	0.10	2672	2432.66
10	1.0	0.5	2.0	0.10	3100	2713.16
11	0.5	1.0	2.0	0.10	2668	2585.16
12	1.0	1.0	2.0	0.10	2668	2588.66
13	0.5	0.5	5.0	0.10	3060	2973.83
14	1.0	0.5	5.0	0.10	2980	3609.33
15	0.5	1.0	5.0	0.10	2980	3017.33
16	1.0	1.0	5.0	0.10	3832	3375.83
17	0.25	0.75	3.5	0.075	2296	2574
18	1.25	0.75	3.5	0.075	3136	3336.83
19	0.75	0.25	3.5	0.075	3472	3462.83
20	0.75	1.25	3.5	0.075	2704	3192.83
21	0.75	0.75	0.5	0.075	1156	1852
22	0.75	0.75	6.5	0.075	5060	4843.5
23	0.75	0.75	3.5	0.025	4052	4439.5
24	0.75	0.75	3.5	0.125	2732	2824
25	0.75	0.75	3.5	0.075	3536	3202.66
26	0.75	0.75	3.5	0.075	2900	3202.66
27	0.75	0.75	3.5	0.075	3248	3202.66
28	0.75	0.75	3.5	0.075	2884	3202.66
29	0.75	0.75	3.5	0.075	3152	3202.66
30	0.75	0.75	3.5	0.075	3496	3202.66

ANOVA showed that the model was very significant as the very high value of the F model (7.31) at a significant level of 1% eliminating the chance of noise. These considerations suggest good suitability of the second-order polynomial model designed to explain observed yields. The coefficients of the surface response model as outlined in equation (3) were evaluated. The student test showed that the two linear coefficients (C and D) and their interactions (CDs) were very significant, their values were P-values, P_C = <0.0001, P_D = 0.0007 and P_{CD} = 0.0026 respectively. However, to minimize errors, all coefficients were included in the model.

Total Carotenoids = (+235.18) + (2614.67 * Glycerol) – (502.67 * Potassium Nitrate) + (1094.15 * Meat Extract) – (5080.00 * Ferric Citrate) – (1108.00 * Glycerol * Potassium Nitrate) + (236.67 * Glycerol * Meat Extract) – (4920.00 * Glycerol * Ferric Citrate) –(72.67 * Potassium Nitrate * Meat Extract) + (7560.00 * Potassium Nitrate * Ferric Citrate) – (11086.67 * Meat Extract * Ferric Citrate) –(987.33 * Glycerol^2) + (500.67 * Potassium Nitrate^2) + (16.13 * Meat Extract^2) + (1.71667E+005 * Ferric Citrate^2)----------(3)

Table 6: ANOVA table for RSM (Coefficient of Determination (R2) = 0.8722, Adjusted R2 = 0.7529 and Predicted R2 = 0.3319* Significant at 1% level)

Source	**Sum of Square**	**df**	**Mean Square**	**F-value**	**p-value prob> F**
Model	0.00000021	14	0.00000156	7.31	**0.0002***
A-Glycerol	0.0000871	1	0.0000871	4.08	0.0617
B-Potassium Nitrate	0.00001094	1	0.00001094	0.51	0.4853
C-**Meat Extract**	0.00000013	1	0.00000013	62.82	**<0.0001**
D-**Ferric Citrate**	0.00000391	1	0.00000391	18.32	**0.0007**
AB	76729	1	76729	0.36	0.5579
AC	0.0000126	1	0.00001260	0.59	0.4544
AD	15129	1	15129	0.07	0.7938
BC	11881	1	11881	0.05	0.8168
BD	35721	1	35721	0.17	0.6884
CD	0.00000276	1	0.00000276	12.94	**0.0026**
A^2	0.00001044	1	0.00001044	0.49	0.4951
B^2	26857.19	1	26857.19	0.13	0.7279
C^3	36125.76	1	36125.76	0.17	0.6867
D^2	0.00003157	1	0.00003157	1.48	0.2429
Residual	0.00000320	15	0.00002137		
Lack of Fit	0.00000281	10	0.00002810	3.56	**0.0869**
Pure Error	0.00003949	5	78986.67		

The Lack of Fit F-value of 3.56 implies that it is not significant relative to the pure error. There is an 8.69% chance that this large Lack of Fit F-value could occur due to noise. A non-significant lack of fit is good, as we want the model to be fit.

Adequate Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. In our experiment, we found a ratio of 10.869, which indicated an adequate signal for the model. Therefore, this model was used to navigate the design space. ANOVA test showed that the coefficient of determination (R^2) for the production of total carotenoids were 0.8722 (Table 6), implying that 14.04% variance occurred could be explained by the model. The R^2 value should be between 0 and 1. The closer the R^2 to 1, the stronger the model, and the better it predicts the response. The predicted R^2 of 0.3319 was in reasonable agreement with the adjusted R^2 of 0.7529. This indicated a good agreement between the experimental and predicted values of carotenoids production.

3D Response Surface plots

The 3D surface response plots were generated for the response (TC) at any two independent variables while keeping the others at their 0 levels. Thus, six 3D plots were obtained by considering all the possible combinations (Fig. 4 a–e). Figure 4 reveals that with increase in glycerol (0.5 g/L), TC production also increases, whereas at higher potassium nitrate (0.5 g/L) and combinations of both, glycerol and potassium nitrate, it leads to decrease in TC production. Figure 4b represents meat extract enhanced increase in TC production. TC production was higher at a combination of glycerol (0.5 g/L) and meat extract (2 g/L) with a comparatively higher slope than only glycerol had. Figure 4c depicts the interaction of ferric citrate and glycerol. With an increase in the concentration of both the variables, TC production was increased and with a further increase in ferric citrate concentration and a mixture of both more than a central point, TC production decreased. Figure 4d represents the effect of meat extract, potassium nitrate, and their interactions. It is evident that meat extract increased the TC production and potassium nitrate led to a decrease in TC production. Interaction with low concentrations of potassium nitrate (0.05 g/L) and higher concentration (above the center point) of meat extract (2 g/L) resulted in increased productivity with higher slop. It could be depicted from Figure 4e that the variables, ferric citrate, and potassium nitrate, were showing the least variation with an increase in concentrations, both have a negative effect on TC. Figure 4f divulges the effect of meat extract, ferric citrate, and their interaction on the product. Meat extract illustrates the highest positive effect on TC production with increasing concentration, whereas at a lower concentration of ferric citrate, TC increases.

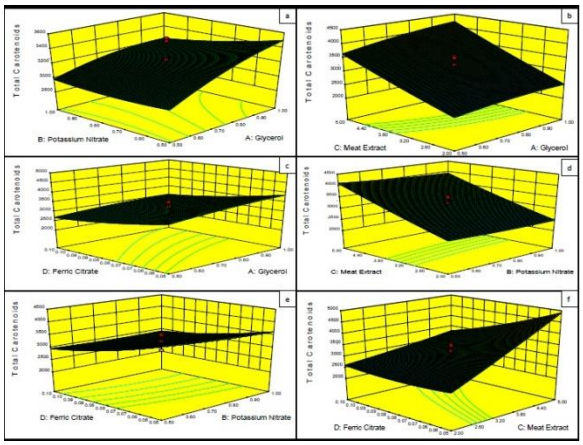

Fig. 4. 3-D response plot of *Paracoccus* OC1 on total carotenoids (TC) (a) effect of glycerol and potassium nitrate on TC, (b) effect of glycerol and meat extract on TC, (c) effect of glycerol and ferric citrate on TC, (d) effect of potassium nitrate and meat extract on TC, (e) effect of potassium nitrate and ferric citrate on TC and (f) effect of meat extract and ferric citrate on TC.

Validation of model

To confirm the predicted response from the medium composition obtained, the statistical model, as well as the regression equation, were validated under optimum conditions. It was found that total carotenoids production was increased and found maximum on 7th day, 5975 ± 346 mg/L, which was near to predicted value of 5404 mg/L with 101.54% yield of carotenoids and indicated that the model was validated for significant response near the predicted value.

DISCUSSION

The genus *Paracoccus* is metabolically versatile containing $C_{18:1}\omega7c$ as a major component of the cellular fatty acids, a gram-negative, catalase, and oxidase-positive and can grow at high-stress conditions [34]. Carotenoid containing *Paracoccus* strains were isolated from soil, as a contaminant from a nutrient agar plate, sediment, and seawater [24, 25, 34, 35]. In the present study, carotenoid producing *Paracoccus* spp. OC1 was isolated from a marine source. The

marine environment became a focus of natural products, because of its relatively unexplored biodiversity compared to terrestrial environments. The potential of marine natural products as pharmaceuticals was introduced by the pioneering work of Bergmann in the 1950s [36]. Marine microorganisms have not only been a tremendous source of biodiversity and chemical diversity but also have capacities to produce highly complex molecules with industrial-scale production of drugs [5, 37]. These novel chemicals from marine organisms that have demonstrated potential as new treatments for cancer, infectious diseases, and inflammation, suggest that there needs to be a greater focus on the development of pharmaceutical and nutraceutical products from marine sources [37].

The microbial system is easier to maintain and scale up than a plant system. The expanding market demand for carotenoids from natural sources has promoted the development of a large-scale production using on hand microorganisms as well as exploring newer sites to find outsources for such a valuable product with a decrease in production costs. Also, by changing physical parameters like medium strength, pH, temperature, and carbon source led to increase in carotenoid production. This is more beneficial for the industry than manipulation in the genetic makeup of an organism. A statistical design like the Plackett-Burman design is not only useful for screening of effect of components on the product, but also to minimize workload and the most precious time. After screening, to settle on the concentration of components for enhancing productivity as well as low cost medium formulation, response surface methodology is the most important [22]. The present study showed a sequential increase in TC production during stepwise optimization; initially, TC production was 1333.33 ± 577.35 mg/L after optimization of basic parameters like medium strength, pH, temperature, aeration, and basic sugar. The maximum nutrient supports the maximum growth of cells but does not support maximum secondary metabolites production. By comparing the yield factors (Ypx, mg of TC produced per gm of cell weight) of media, 10 times diluted media had the highest yield. Starvation of the nutrient supplement suppressed the cell division and enhanced carotenoids production. Results obtained are in corroboration with the Ibrahim, who reported that the MB1/10 media supports the highest carotenoid production [38]. The favorable pH for the growth of *Paracoccus* sp. OC1 was pH 8 which could be due adaptation of organism in the seawater with pH 8. However, Attri et al., [39] reported that medium of pH 6 supported the highest carotenoid production and acidic pH favors β-carotene production in *Monascus* sp. and in *Rhodotorula* sp. [40]. The present study showed the highest carotenoid production at neutral to slightly alkaline pH. Ibrahim [38] reported that the optimum temperature for carotenoid production for *Micrococcus* sp. was 30°C whereas 37°C was the optimum temperature for the highest biomass accumulation. These observations are in corroboration with our results for growth of OC1. However, the highest TC production was obtained at 37°C instead of 30°C. The temperature of the Gulf of Khambhat was also near to 25-30°C from which this strain was isolated and it may be required for the growth and activation of different enzymes involved in carotenoids biosynthetic pathway. At temperature of 45°C, the denaturation of enzyme structure might be the reason for decrease in carotenoids accumulation. Highest biomass and carotenoid production at 29-32°C were reported in *Rhodotorula glutini* [41], whereas higher β-carotene accumulation at 20°C was reported in *Phaffia rhodozyma* than 30°C [42]. This clearly indicates the strain specific effect of temperature on carotenoid production. The addition of glycerol supports higher β-carotene production in metabolically engineered *Escherichia coli* [9]. Glycerol can convert into pyruvate and acetyl CoA, both of which are precursors for the tetraterpenoid pathway. Thus glycerol enhances the accumulation of precursor that leads to higher carotenoid production. The addition of glucose, maltose, and sucrose leads to a decrease in carotenoid production as compared to glycerol. Supplementation of medium with glycerol and glycine resulted in higher total carotene accumulation in *Chlamydomonas acidophila*, an extremophile microalga [26]. With an increase in aeration, the color of pigment was changed from orange to dark brown. The higher carotenoid production with intensive aeration (1.3 L/L. min)

was observed in *Rhodotorula rubra* GED2 and *Lactobacillus casei* sub sp *casei* [15]. Aeration leads to pathway extension by incorporating oxygen molecules in the skeleton of the carotenoid molecule and can drive the pathway up to xanthophylls class.

The metabolic and genetic engineering approach is used for higher microbial carotenoid production but requires highly urbanized technology [43]. By changing nutritional parameters, the metabolite production can be increased and it becomes commercially feasible due to low-cost technology. Initial screening of the ingredients was done to understand the significance of their effect on the product formation and then a few better ingredients were selected for further optimization. Choudhary and Singhal [44] also used a statistical approach to enhance β- carotene production from *Blakeslea trispora*. Plackett-Burman design showed only glycerol, meat extract, ferric citrate, potassium nitrate, and ammonium nitrate had a positive effect on TC. Marine broth contains a peptic digest of animal tissue and the supplement of glycerol, which supports the highest carotenoid production. This supports our observations as both of these compounds had a positive effect on TC [9, 38]. Presence of inorganic salts like magnesium, manganese, calcium, zinc, potassium, and ferrous as a medium supplement showed a higher rate of carotenogenesis by serving as a cofactor of key enzymes like phytoene synthase [45]. In the present study also showed, isolate OC1 required a basal level of ferrous and potassium for carotenoid production. *Haematococcus pluvialis* showed the stimulation of phytoene synthase and carotenoid hydroxylase levels when grown in the presence of increased light intensity together with sodium acetate ferrous salt [46, 47]. Hyper-accumulation induced by ferrous ion was due to the generation of hydroxyl radical, which motivate cellular carotenoid synthesis, from the Fenton reaction ($H_2O_2+Fe^{2+}\rightarrow Fe^{3+}+HO^{-}+HO^{*}$) [48]. *Chlorococcum* and *Rhodotorula* strain also showed improvement in carotenoid yield in the presence of inorganic salts [49–51]. Basic cellular metabolic pathways like the tricarboxylic acid (TCA) cycle and its intermediates play an important role in carotenoid and lipid biosynthesis in microbes. Initially, citrate and malate were major components answerable for inspired carotenogenesis in *Rhodopseudomonas spheroids.* Later on, in *Blakeslea trispora* and *Xanthophyllomyces dendrorhous* also found carotenoid accumulation in the presence of citrate [52]. In *Sporobolomyces roseus*, cell growth and β-carotene production were observed when the medium was supplemented with succinate. Another report on carotenoid synthesis due to the presence of 0.2% (w/v) malate, citrate, α-ketoglutarate, fumarate, or oxalacetate in *Actinomyces crysomallus* was examined. Most of the *Paracoccus* species required nitrate as a final electron acceptor and can reduced nitrate into N_2 [53].

CCD with RSM is only applicable to a small number of variables (up to five) [22]. In the present study, four components were screened and achieved TC production range 1156 mg/L to 5884 mg/L using *Paracoccus* OC1 isolate, which was five-fold higher as compare to the previous optimization of fermentation parameters. A similar increase in reported 22.6% higher productivity of carotenoids in *Xanthophyllomyces dendrorhous* through statistical approach by Plackett-Burman and Uniform experiment design [54]. The medium optimization results suggest that carotenoid biosynthesis in *X. dendrorhous* is promoted by high C/N ratio+, low carbon and high nitrogen concentrations, and a slightly acidic condition when the cell growth is suppressed, whereas in *Paracoccus* sp., carotenoids production was only increased at low carbon and nitrogen concentration. A higher concentration of the nutritional medium components leads to cell growth but does not provide enough stress that is required for secondary metabolites (carotenoids) production. Wang et al., reported an increase of β-carotene (13.43 mg/L) yield, 34.17% compared to the control [55], and Malisorn and Suntornsuk also showed 2.7 g/L biomass and the maximum β-carotene of 201 μg/L, approximately 15% higher by response surface methodology (RSM) approach with a factorial design and CCD in *Rhodotorula glutinis* [56].

Commercially available carotenoid producing microorganisms can synthesize β-carotene, *Dunaliella salina* (10.35 mg/L), *Mucor rouxii*

(31.0 μg/g), *Dunaliella bardawil* (35.5 pg/cell), *Rhodotorula glutinis* mutant 32 (250 mg/L), *Blakeslea trispora* (420 μg/g) and *Phycomyces blakesleeanus* (1200 μg/g) whereas our isolate OC1 was able to produce 5884 mg/L. Previously *Paracoccus* strain is reported for astaxanthin production [24] whereas we report novel *Paracoccus* strain OC1 having the capacity to produce β-carotene and the first report on optimization using a statistical approach. This was evident in amplification of the genes involve in the production of caretenoids. β-carotene hydrolase and β-carotene ketolase genes were not amplified in the PCR reaction which suggested the absence of gene and due to this reason our isolate cannot produce astaxanthin. This result supported our previous results that extracted carotenoid was β-carotene and our isolate is a novel β-carotene producing *Paracoccus* strain.

The carotenoid biosynthetic pathway has been studied extensively. In the β-carotene biosynthetic pathway, phytoene synthase, phytoene desaturase, and lycopene β-cyclase genes are involved [57, 58]. Subsequently, β-carotene is used to synthesize xanthophyll types of carotenoids. Previously, *Paracoccus* species were reported for astaxanthin production [24] but our isolate produced only β-carotene. To prove this the presence of genes encoding key enzymes of the carotenoid biosynthetic pathway was amplified. Lycopene β-cyclase is an enzyme for the production of carotene molecule, β-carotene hydrolase, and β-carotene ketolase are two key enzymes responsible for the pathway driven towards xanthophylls [59]

CONCLUSION

Paracoccus strain is known for xanthophylls type of carotenoid production, whereas we have isolated and characterized a β-carotene producing strain which is confirmed by the amplification of the genes involved in the carotenoid production. Application of statistical methods for screening and optimization of the medium components successfully improve total carotenoid production.

Acknowledgments: Authors are thankful to Gujarat Council on Science and Technology (GUJCOST), Gujrat, India for the financial aid (File Number: GUJCOST/MRP/2014-15/1243). The authors are also thankful to Charotar University of Science and Technology (CHARUSAT) management for providing necessary facilities.

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PRAJÑĀ **- Journal of Pure and Applied Sciences Vol. 27-28: 41 - 52 (2019-20)**
ISSN 0975 - 2595

ANTIOXIDANT AND QUALITY ATTRIBUTES OF *DAHI* FORMULATED USING POTENTIAL PROBIOTIC STRAIN ISOLATED FROM INFANT FECAL

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ABSTRACT

Objective of the present study was to formulate dahi using potential probiotic strains isolated from infant fecal as well as to study their antioxidant, physicochemical, microbiological, and sensory properties. Three lactic acid producing species were isolated from infant fecal. Three different dahi samples (EX-I, EX-II and EX-III) were prepared using isolated strains. The EX-IV dahi was prepared using combination of isolate II and III. From the samples pH, acidity(%), synersis(%), and total solids(%) were estimated and the range were 3.83-5.00, 1.25–2.04, 22.33–29, and 12.40–18.00%, respectively. The total phenol was highest (11.39 mgGAE/100g) in EX-I dhai. The total antioxidant capacity (FRAP & ABTSRSA) was found higher in EX-IV compared to all other samples. The LAB count was highest in EX-II and EX-IV (≤9 log cfu/g). The highest sensory score was obtained for control followed by EX-I. In conclusion, the isolated potential probiotic strains could be used for functional food product development.

Keywords: isolated probiotic strain, dahi, antioxidant, microbial

INTRODUCTION

Dahi is an indigenous Indian fermented milk product known for its stimulating taste, palatability and curative values; it also called as 'curd' **[1]**. *Dahi* is a popular fermented milk product in India consumed in almost every household **[2]** by all age groups from infancy to geriatrics. It is prepared from buffalo milk, cow milk or standardized milk **[2]**. As per PFA rules**, "***dahi* or curd is a product obtained from pasteurized or boiled milk fermented with a culture". The different starter culture used in the manufacture of commercial *dahi* namely *Lactococcus. lactis, Streptococcus thermophilus, Lactobacillus bulgaricus, Lactobacillus. Plantarum, Lactobaccilus. cremoris,* and lactose fermenting yeasts **[3]**. Around 90% of the total fermented milk products produced in India is in the form of *dahi* **[2]**. Among the various cultured milk products, the international market for *dahi*/curd and chilled desserts has been benefited due to extensive growth toward healthy food and due to new product development **[2]**. From the last decade demand for *dahi*/curd has increased greatly, because of it's nutritional and therapeutic benefits **[2]**.

Although in the traditional practice lactic acid bacteria (LAB) involves as a starter cultures for milk fermentation, they can also be serve as protective cultures against microbial pathogens

and spoilage organisms in minimally processed foods **[4]**. Some lactic acid bacteria act as important probiotic because of their strain-specific properties that are beneficial to health **[5]**. To action as probiotics, bacterial strains should meet certain requirements such as tolerance to high acid and bile environment **[6]** and many more. Fermented milk products with probiotic organisms have gain special attention due to their confirmed health claims**[7].**

In recent time, natural antioxidants from natural sources have gain popularity over synthetic one. Protein hydrolysates and peptides from different plant and animal protein showed good antioxidant and ACE inhibition capacity**[7].** Various studies reported a proteolytic process during fermentation of milk by lactic acid producing bacteria. Thus, the need of probiotics with antioxidant activity to minimize oxidative stress is a new alternate to maintain a good health**.**

The ecosystem of gastrointestinal microflora is a very complex [**8]**. within the intestinal microflora more than 400 species identified and many of them population levels nearly as high as 10^{12} per g in the colon [**7]**. Initial colonization of the aseptic intestine of the newborn happens during birth when bacteria from the mother's intestinal and vaginal flora inoculate the gastro-intestinal tract of the fetus **[9].** Lactobacillus strains are found naturally in the human intestine; therefore, such strains are specially developed for commercial use as probiotics [**10**]. Some researchers reported that bacteria isolated especially from the feces of infants or elderly humans possess potential probiotic properties [**10**]. Now days, probiotics are known as potential, novel and natural therapeutic drugs [**11**]. Thus, the isolation and characterization of new strains are still needed [**12]**.

Thus, the aim of this study was to isolate lactic acid strain from infant fecal sample and formulation of *dahi* using isolated lactic acid strain as well as to evaluate physicochemical, antioxidant, microbiological, and sensory properties of *Dahi* compared to market dahi.

METHODOLOGY

A. Isolation of strains

An eight-month-old infant (healthy and without any prenatal problems) was a donor. The fecal sample was collected in a sterile container. Five gram of the feces was suspended and homogenized in 45 ml of phosphate buffered saline (PBS) pH 7.4. The homogenized feces were used to prepare the 10-fold serial dilutions. 0.1ml of an appropriate dilution were spread-plated on Man, Rogosa and Sharpe agar (MRS) and incubated at 37°C under anaerobic conditions in an anaerobic jar. After 48 hour of incubation, three colony was based on size selected among them two were big and one was small in size. All lactic acid bacterial isolate were inoculated in MRS broth at 37°C for 24 hour. The culture stock was prepared in 50% glycerol and stored at -20°C until use.

To prepare the starter culture, the broth culture was centrifuged at 8000 rpm for 15 min, and the

pellet was washed twice with 0.9% NaCl and 1ml of washed culture were suspended in sterile skim milk tubes. These tubes were incubated overnight at 37°C, subsequently in the skim milk successive five transfers of the cultures were given before used as a starter culture for *dahi* preparation

B. Formulation of *Dahi*

400ml of UHT treated toned milk was purchase from Amul outlet (Anand). Milk was heated till obtained 45°C. Milk was divided into four equal portions (100ml x 4) and in the aseptic condition 2% of activated cultures of isolated strain were added into each 100ml of milk. The samples were incubated at 42°C until the coagulation completed (17 to 22 hrs). Cooled the samples at 4°C for 4hr. Four samples were prepared using 3 isolated strains. Three experimental *dahi* samples were cultured using three different isolates (i.e EX-I,EX-II and EX-III) and fourth *dahi* sample was cultured using combination of isolate II & III (EX-IV). Market *dahi* was used as control sample.

C. Parameter studied

Experiments were carried out in duplicate. *Dahi* samples were collected and subjected to physicochemical, Antioxidant, microbiological, and sensory properties.

i. Physicochemical Properties

pH was measured using a pH meter (Systonic) after pH calibration with standardized solutions to pH 4 and 7. Titratable acidity in % lactic acid was measured according to **BIS [13]** method. The syneresis of *dahi* samples was determined as described by **Prasanna et al. [14]**. The total solids and moisture content of all the samples was determined according to the Association of Official Analytical Chemists method **[14]**.

ii. Antioxidant Properties

Total phenol content was measured with Folin-Ciocalteu's phenol reagent using the method described by Cho et al. [**16**]. For comparison gallic acid was used as a standard and the results are expressed in mg GAE/100 g.

Total antioxidant capacity of the *dahi* samples were determined by using ferric reducing antioxidant power assay (FRAP) [**17**] where trolox was used as standard for the comparison and results are expressed as mg TE/100 g while DPPH (2, 2-Diphenyl-1-picrylhydrazyl) and ABTS [2, 2 Azinobis (3–ethylbenzothiazolin-6-sulfonic acid) diammonium salt] radicals scavenging activity were determine as described by Brand-Williams et al. [**18**] and Re et al. [**19**], respectively where percentage inhibition was calculated and results are expressed as mg TE/100 g sample.

iii. Microbiological Assay

10 g of the *dahi* samples was collected aseptically and blended using a stomacher (Minimix 100Pc) with 90 ml of sterile 0.1 % peptone water then submitted to serial dilutions with the same diluent. The count of isolated probiotic strain was estimated by spreading 0.1 ml of each dilution, respectively in petri-dish

containing DeMan-Rogosa-Sharpe agar (MRS agar). Yeast and mold, *E. coli* and total coliform population were enumerated on Potato Dextrose agar, Hi-chrome agar and violate red bile agar (VRBA), respectively. Petri plates were incubated at 37 °C for 48 h. At the end of incubation colonies were counted and the results expressed in log cfu/g.

iv. Sensory Evaluation:

The acceptability of the experimental and control *dhai* was assessed by the modified hedonic score card where a panel of six judges tasted and scored the product on a scale of 1-9, where 1 = extremely dislike and 9 = extremely like for the sensory attributes of odor, texture, syneresis, acidity, taste and flavor as well as for overall acceptability .

D. Statistical analysis:

All the data were expressed as Mean ± SD. To analyze the results, one-way analysis of variance (ANOVA) and Duncan test ($p \leq 0.05$) were used. Statistical analysis was performed using the SPSS 20 version.

RESULTS AND DISCUSSION

Among the selected three isolates on MRS agar, morphologically two big size colonies were rod shape and one small size colony was cocci, all were gram positivity, and catalase negativity, all isolates were identified as lactic acid bacteria through biochemical test. These strains were also analyzed for probiotic potential and results showed that all three strains were tolerated pH3 and 1% bile. The data was not discuses in this paper.

i. Physicochemical Properties

The pH of control and experimental samples ranged between 3.88 to 5.00 (table 1) where EX-IV showed significantly ($p \leq 0.05$) lower pH and EX-II showed significantly($p \leq 0.05$) higher value compared to control and EX-I and EX-III *dahi* samples. The optimum pH of market thick fermented milk is from 3.27 to 4.59 [**15]**, in the present study pH of *dahi* samples also fell into this optimum range except pH of EX-II *dahi*. Similar range of pH also reported by **Rasdhari et al. [20], Patel et al. [21]**, **Sudhir et al. [3]** and **Cho et al. [16] in** their studies.

Values of acidity (%) were observed inversely proportional to the pH value of *dahi* samples. Control and experimental *dahi* samples acidity were ranged from 1.25 to 2.51 % (table 1) where EX-IV showed significantly($p \leq 0.05$) higher acidity and EX-II and EX-III showed significantly($p \leq 0.05$) lower acidity. Similar range of acidity also reported by **Rasdhari et al. [20], Sudhir et al. [3] and Cho et al. [16]** in their studies i.e. 0.81 to 0.83%, 0.52 to 1.26 and 0.92 to 0.94%, respectively.

The synersis of all experimental *dahi* samples except EX-II showed significantly($p \leq 0.05$) lower values (22.33 to 26.00%) compared to control *dahi* (29.00%). According to **Fox et al., [22]** Syneresis is directly affected by acidity and is inversely proportional to pH. This statement was also true for this study. Synersis (%) content of control and experimental samples ranged between 18.25 to 22.5 % by **Rasdhari et al. [20]**

and 14.28 to 25.55 by **Cho et al. [16].** In the present study similar values were observed for sample EX-I, EX-III and EX-IV while control and EX-II showed slight higher synersis (%).

Total solids (%) and moisture (%)content of control and Experimental *dahi* samples were ranged from 12.40 to 18.00 and 82.00 to 87.60(%), respectively (table 1), where control *dahi* showed significantly ($p \leq 0.05$) higher total solids content and EX-II showed significantly ($p \leq 0.05$) lower total solids content among all the *dahi* samples. Results of moisture content were inversely proportional to results of total solids content. Similar range of total solids content was also observed by **Rasdhari et al. [20]** and **Sudhir et al. [3]** i.e 24.85 to 25.47% and 23.87 to 36.37%, respectively.

Among all the experimental *dahi* samples isolates I (EX-I) and combination of isolate-II and III (EX-IV) took less time (i.e 17hr) for coagulation while when isolate –II and III add singly it took higher time (i.e 22hr) for coagulation. They also shown a lower acidity compared to combination (i.e EX-IV).

ii. Antioxidant Properties

Probiotic organisms act as an antioxidant by changing the redox status of the host by metal chelating, antioxidant capacity, regulating signaling pathways, inhibiting enzymes producing ROS etc. [**23**] while total phenolic content (TPC) present in milk may be due to the presence of polyphenols in milk, that mostly come from the feed **[24]** as well as because of the protein and reducing compounds **[25]**. The various microbiological cultures degrade the such primary components of milk such as protein, carbohydrate and lipids into various secondary forms like free amino acids, peptides, organic acids, free fatty acids, where the free amino acids and peptide sequences owns antihypertensive effect **[26]**, increasing of antioxidant capacity and inhibition of lipid peroxidation [**27]** this functions can be very beneficial for the human health and wellbeing. It is reported that various strains of lactobacillus showed antioxidant capacity in the yoghurt [**23**]. Hence in this study antioxidant capacity and total phenol were measured.

Mean values of total phenol content, and total antioxidant activity measured using FRAP, DPPH and ABTS can be seen in Table 2.

The mean values of total phenolic content equivalent to gallic acid of control and experimental *dahi* samples ranged between 4.53 to 11.39 mg/100 g (table 2) where control *dahi* showed significantly ($p \leq 0.05$) lower total phenol content compared to all experimental samples, Ex-I *dahi* showed significantly ($p \leq 0.05$) higher (11.39 mg/100g) total phenol content followed by EX-III *dahi* sample (10.29 mg/100g). Similar result was reported by **Cho et al. [16]** i.e 4.3 to 6.9 mg GAE/100g while **Sudhir [3]** and Dabija et al. **[7**] reported higher

Total phenol content, 218 mg/100g and 0.99 GAE/g, respectively compared to results of the present study.

FRAP assay has been reported to be suitable to measure antioxidant activity of substances having half-reaction redox potential below 0.7 V. This measures only non-protein antioxidant capacity. Milk component such as urate, ascorbate, alpha-tocopherol and bilirubin have been characterized to have ferric reducing ability [**28**]. The total antioxidant capacity estimated by FRAP assay measures the effect of antioxidant of any substance in the reaction medium as reducing ability of the complex Fe(III)-TPTZ (2,4,6-tris(2-pyridyl)-s-triazine) [**29]**. Ferric reducing antioxidant power of control and experimental samples ranged between 27.50 to 36.32 mg TE/100 g (table 2), where EX- IV followed by EX-I *dahi* sample showed significantly ($p \leq 0.05$) higher value (36.32 and 34.72, respectively) compared to EX-III (27.5 mg TE/100 g) *dahi*. Compared to the present study **Sudhir [3]** reported lower FRAP content (111 µM trolox/g) while **Chouchouli et al.[25]** reported higher FRAP content (96mg AAE/100g) in their study.

The DPPHRSA assay is a simplest method which gives information on the radical scavenging activity of the antioxidant substances present in a sample **[28]**. The mean value of DPPHRSA of control and experimental *dahi* samples ranged between 2.64 to 7.09 mg TE/100 g (Table 2) where control *dahi* showed significantly ($p \leq 0.05$) higher (7.09 mg TE/100g) value compared to all experimental *dahi* samples. Similar range of DPPHRSA also reported by **Sudhir et al. [3] and cho et al. [16]** which were 53 µM trolox/g and 42 to 47% inhibition, respectively. While **Dabija et al. [7]** obtained lower DPPHRSA (10.56% inhibition) compared to present study.

The antioxidant capacity of foods can be measured using autobleaching of a preformed solution of ABTS radical cation **[29]**. The decolourization of the solution takes place in the presence of hydrogen donating antioxidant (nitrogen atom quenches the hydrogen atom) [**29].** The ABTS radical scavenging activity was found in the range of 3.52 to 5.48 mg TE/100 g (Table 2) where EX-II *dahi* showed significantly ($p \leq 0.05$) higher (5.84 mg TE/100g) value and control *dahi* showed significantly($p \leq 0.05$) lower (3.52 mg TE/ 100g) value compared EX-I, EX-III and EX-IV *dahi* sample. Compared to the present study **Sudhir et al. [3]** reported higher ABTSRSA (245µM trolox/g).

iii. Microbial Analysis

Microbiological characteristics are indicators of quality, safety and shelf-life of the prepared product. For the microbial analysis of *dhai* samples, probiotic lactic acid bacterial were enumerates on MRS agar and contamination bacteria such as *E.coli*, total coliforms and yeast and mold enumerated on *E.coli* Hi-crome agar, Violate red bile agar and potato dextrose agar, respectively.

The mean value of probiotic lactic acid bacterial count was ranged between 6.05 to 9.20 log cfu/g (Fig;1) where control *dahi* sample showed zero count. Among experimental sample EX-I had significantly ($p \leq 0.05$) lower count compared to EX-II and EX-IV. The highest count was found in EX-IV. No growth in control *dahi* could possible due to commercial cultures as this *dahi* sample is commercially produced. FAO/WHO recommendation that if food sold with health claims from the addition of probiotics it must contain, per gram, at least 10^6 to 10^7 cfu/ml of viable probiotic bacteria **[30]** and in the present study all experimental samples fulfill this criterion. Various researchers reported lactobacillus count 7.00 to 9.38 log cfu/ml in the fermented milk (**[16], [3] and [20]**).

Contamination of *E.coli*, total coliforms and yeast and mold were observed in any experimental samples while control sample showed contamination of *E.coli* and total coliforms (3.21 and 3.10 log cfu/g) (Plate:1). Yeast and mold contamination was not found in any of the *dahi* samples .

iv. Sensory evaluation

Sensory evaluation of dahi sample was carried out using modified hedonic card for the different sensory attributes such as odor, texture, synersis, acidity, taste and flavor as well as for over all acceptability. Only two experimental samples were analyzed for sensory attributes i.e. EX-I and EX-IV, which take lower coagulation time (i.e 17hr).

The mean score of odour, texture and synersis were ranged between 6.20 to 7.40 and statistical analysis showed no significant difference between control and experimental *dahi* samples (Fig:2). The value of the pH and acidity was effect the sensory perception of *dahi* samples. The acidity values EX-IV *dahi* sample was higher which affect on the sensory perception and this sample showed significantly lower score for acidity, taste and flavor as well as for overall acceptability(OA) (5.6,6.0, and 6.2, respectively). Control sample obtained higher score for all the sensory attributes (7.2 to 8) followed by EX-I (6.8 to 7.2) *dahi*

CONCLUSION

From the results obtained it is concluded that the isolated strains posses good antioxidant properties and formulated a good quality curd. Hence, they could be further used as a potential probiotic culture to produce milk fermented products and also to prepare non- dairy fermented foods or synbiotic foods.

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Table 1: Physicochemical properties of control and experimental *dahi.*

Samples	pH	Acidity (%)	Synersis (%)	Total solids (%)	Moisture (%)	Coagulation time (hr.)
C	4.22±0.00^{b,c}	1.45±0.01^{b}	29.00±0.00^{c}	18.00±0.00^{d}	82.00±0.00^{a}	--
Ex-I	4.00±0.00^{b}	2.04±0.05^{c}	22.33±0.58^{a}	13.40±0.00^{b}	86.60±0.00^{d}	17
Ex-II	5.00±0.00^{d}	1.25±0.02^{a}	28.67±0.58^{c}	12.40±0.00^{a}	87.60±0.00^{d}	22
Ex-III	4.66±0.00^{c}	1.34±0.02^{a}	26.00±0.00^{b}	13.00±0.00^{b}	87.00±0.00^{c}	22
Ex-IV	3.83±0.00^{a}	2.51±0.11^{d}	22.33±0.58^{a}	14.67±0.58^{c}	85.33±0.58^{b}	17
F- value	264.2*	256.47*	159.167*	253.36*	253.36*	--

Mean ±SD of three trials
Means carrying different superscripts within a column are significantly different at $p \leq 0.05$
*indicate significant difference ($p \leq 0.05$)

Table 2: Total phenol content and total antioxidant capacity of control and experimental *dahi.*.

Samples	Total Phenol (mg GAE/100 g))	Total antioxidant capacity (mg TE/100 g)		
		FRAP	DPPHRSA	ABTSRSA
C	4.53±0.23^{a}	31.53±0.97^{a,b}	7.09±2.86^{b}	3.52±0.12^{a}
Ex-I	11.39±0.29^{d}	34.72±4.17^{b}	3.34±0.61^{a}	4.88±0.96^{a,b}
Ex-II	7.55±0.07^{b}	32.01±1.87^{a,b}	3.72±0.83^{a}	5.84±1.55^{b}
Ex-III	10.29±0.15^{c}	27.50±1.39^{a}	2.64±0.77^{a}	5.01±1.04^{a,b}
Ex-IV	7.40±0.39^{b}	36.32±3.4^{b}	4.36±1.16^{a}	5.70±0.80^{a,b}
F- Value	340.62*	4.85*	3.95*	2.08

Mean ±SD of three trials
Means carrying different superscripts within a column are significantly different at $p \leq 0.05$
*indicate significant difference ($p<0.05$)

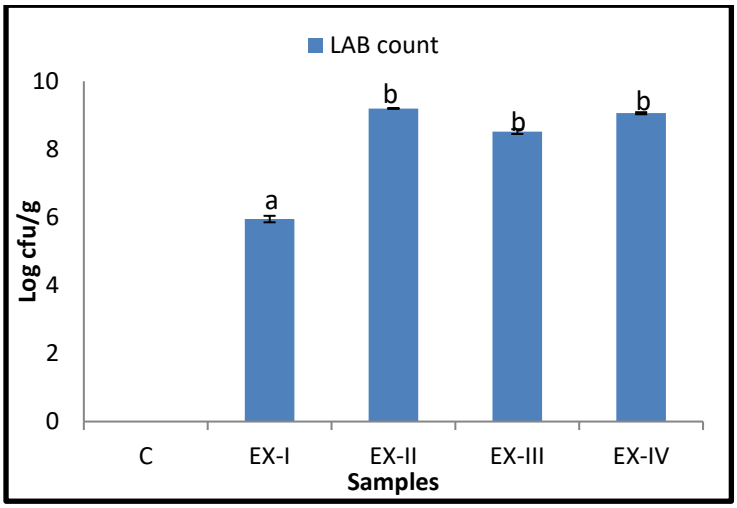

Fig.1: LAB count (log cfu/g) of control and experimental *dahi.*
Mean ±SD of three trials
Means carrying different superscripts are significantly different at $p \leq 0.05$

Plate:1 *E.coli* and total coliforms count on Hicrome *E.coli* agar and VRBA petriplate, respectively.

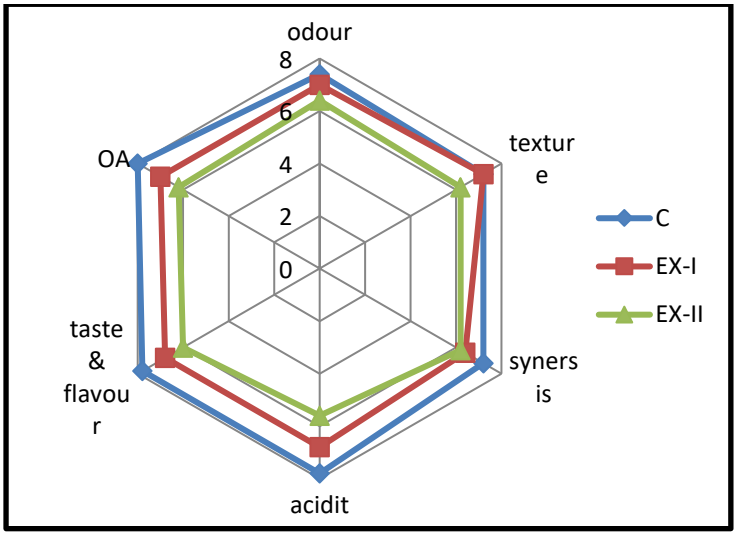

Fig:2 Sensory attributes score of control and experimental *dahi.*

PRAJÑĀ - **Journal of Pure and Applied Sciences Vol. 27-28: 53 - 61 (2019-20)**
ISSN 0975 - 2595

ANTIOXIDANTS AND TYPE – II DIABETES RELEVANT ENZYME INHIBITION OF MOTH BEANS (*Vigna aconitifolia*) GERMINATED WITH ASCORBIC ACID AS AN ELICITOR

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ABSTRACT

This study aims to determine the effect of elicitor on the antioxidant, α- amylase and α- glucosidase inhibition activity of germinated moth beans. The germination was carried out by hydrating the seeds in varying concentration (0-200mg/L) of ascorbic acid. The results showed that the Total antioxidant capacity (TAC) as well as α- amylase and α- glucosidase inhibition activity was increased significantly (P ≤ 0.05) in the germinated samples. The total phenolics, Flavonoid and Proline levels were increased significantly (P ≤ 0.05) in the germinated samples compared to the raw moth beans.Flavonoid, DPPH–RSA and ABTS-RSA also increased in germinated moth beans. The carbohydrate digestive enzymes (α-amylase and α-glucosidase) activity was inhibited significantly in germinated moth beans. The Pearson correlation showed a significant relation between Total phenols and TAC. The findings suggested that the moth beans sprouts may be used as a nutraceutical food with the potential to give the quality of human health and alleviate the effect of prevalent of non-communicable disease.

***Key Words**: Moth beans, Elicitor, Antioxidant, Diabetes mellitus, Ascorbic acid*

INTRODUCTION

Diabetes mellitus, particularly type 2 diabetes (T2-D)with its rapid increasing prevalence become a global public health problem. The pathogenesis and progression of this disease as well as development of secondary complications of T2-D is directly linked to chronic hyper-glycemia and oxidative stress [1].Therefor regulation of blood sugar level after meal by modulating the activities of carbohydrate digesting enzymes such as α – amylase and α – glucosidase is main strategy for controlling chronic hyper-glycemia [2]. Current treatment to control hyper glycemia is the use of synthetic inhibitors to regulate the activity of these enzymes, and to delay the rate of glucose absorption in small intestine [3]. These synthetic drugs inhibit the enzyme but may cause severe side effects such as gastrointestinal distress and hence, search for safe hypoglycemic agents from plant foods which are commonly consumed using in vitro models has advantage [4].

Plant foods containing varieties carbohydrate digestive enzymes namely α-amylase and α-glucosidase and are not showing any side effect [5]. These compounds are also considered as antioxidants therefore, they may provide additional protection against oxidative stress induced by hyperglycemia. The phytochemical composition of plant foods depends on genetics, physiological and agronomical factors which are known as abiotic and abiotic and can be used to increase phenolic compounds in plant foods. These health's related phenolic compounds can be increased in plant systems by stimulating protective endogenous pathways such as redox-linked pentose phosphate pathway (PPP) [6].

Eliciation is a process in which elicitor application can be used to increase metabolite productionin plant and to increase its nutraceutical value for that plant [6] .Various research studies have reported increased phenolic metabolites in legumes in response of elicitor treatment along with germination through PPP associated endogenous defence response [7,8,9]. Oregano extract, Folic acid, and Chitosan are considered as natural elicitors. Ascorbic acid is a strong water soluble antioxidant and induced a stress response in the host leading to increased synthesis of bioactive phenolics, L- DOPA and enhanced antioxidant activity [10]. Germination is a simple, low cost and home scale process which improves nutraceuticals and bioactive compounds of legumes.

MATERIALS AND METHOD

Chemicals: L-Ascorbic acid (95210), DPPH (2,2-Diphenyl – 1-picrylhydrazyl) (D 9132), Gallic acid (G7384),N-(1-napthylethylenediaminedihydrochloride) (N 9125), Rutin hydrate (R 5143), TPTZ (2,4,6-Tris (2-pyridal) -s- triazine) (T 1253) , PPA (porcine pancreatic alpha amylase type VI-B) (A 3176), Acarbose (A 8980), pNPG (4-p nitrophenyl – α- glucosidase (Type I from baker's yeast) (G 5003), were purchased from Sigma Aldrich Company (St. Louis, MO, USA). Soluble starch purchased from SRLMumbai, India. All other chemicals were purchased from local manufacturer and were AR grade.

Seed treatment and germination:

Mothbeans (*Vigna acontilifolia*) were purchased from the D- martstore of V. V. nagar, Gujarat, cleaned and stored in plastic container till further used. The seeds (50 g) were primed in ascorbic acid solution having varying concentration of ascorbic acid(0 to i.e. 0 to 200mg /L. Fifty grams of seeds were placed in 500 ml of solution in 1000ml of conical flasks. Then soaked for 12 hrs. The pre-soaked seeds were washed with distilled water and germinated at 37 ˚C for 24 hrs. between two layers of filter paper and kept moist by sprinkling water at regular time period. These sprouts were dried, powdered and stored at – 20 ˚ C till analysis. The known amount of (3 g.) of powder was extracted

using 15 ml of methanol: water (80:20) solvent with P^H 2. The dried powder was crushed in a motor and pestle. The mixture was kept in shaker for 30 minutes. The content were centrifuged for 10 minutes at 6000 rpm. The supernatant was collected in a sugar tube. To the residue 10 ml of methanol: water was added and process was repeated. The supernatant was pooled and made the volume to 50 ml with the solvent. There extracts were analyzed for biochemical and enzymatic assay.

A. Determination of Total phenols :

The total phenolic content of methanolic extracts of moth (raw and sprouts) was estimated by using Folin- Ciocalteu method and the absorbance was read at 750 nm. [12] and modified by [11].Gallic acid solution of different concentrations treated same as the samples and results were expressed as mg of Gallic acid equivalent (GAE) per 100 g of Sample (mg GAE/100g)

B. Flavonoid:

The Flavonoid content of all extracts was analysed using calorimetric method and the absorbance was read at 510 nm [13]. Rutin was taken as standard and comparison was done. The results were expressed as rutin equivalent (mg RE) / 100 g. of dry samples.

C. Determination of Total Antioxidant Capacity:

Ferric Reducing Antioxidant PowerAssay (FRAP):

FRAP assay was carried out by method developed by Benze and strain [14] and modified by et.al [11]. Trolox standard curve was developed using different aliquots and results were expressed in mg Trolox Equivalent Antioxidant capacity (TEAC) / 100g of moth.

DPPH Radical Scavenging Activity Assay (DPPH - RSA):

The method described by Bran–Williams et. al. [15] and modified by [11] Was followed for determining the antioxidant activity of the methanolic extract of moth, on the basis of the scavenging activity of the stable 2,2- Diphenyl – 1 picrylhydrazyl (DPPH) free radicals. Extract was added to methanolic solution of DPPH and absorbance at 517 nm was recorded after 20 minutes. Percentage inhibitions were calculated using the formula (1):

(A Control- A Sample/ A control)*100 and the results were expressed as mg TEAC /100 g of Moth

ABTS Radical Scavenging Activity Assay (ABTS – RSA):

The free radical – scavenging activity was determined by ABTS (2,2-Azino-bis (3–ethyl benzo thiazoline – 6- sulfonic acid diammonium salt) radical cation decolourization assay

described by RE. et. al. [15 and modified by 11]. The results were calculated using the formula (1) and the results were expressed as % of TEAC of dry weight basis.

D. Proline Assay:

Proline content was determined according to the modified method of Bates.et.al.[16].The concentration of Proline was calculated from a Proline standard curve and expressed as micromole per gram of dry sample.

E. Alpha Amylase Inhibition capacity:

α- amylase inhibition assay was determined by the slightly modified starch- iodine colour change method described by Kotowaroo et. al. 2006, Mahomoodallyet. al, 2012. [18]and modified by Anuet. al.[19]. Here 300 µl of α-amylase solution from porcine origin was added to 1 ml of soluble starch solution and 100 µl of sodium acetate buffer (0.1 M, P^H 7.2). The reaction mixture was incubated for 37 ˚Cfor 1 hr. Then, 100 µl from the reaction mixture was discharged into 3 ml of distilled water and 100 µl of iodine solution. The absorbance of the starch – iodine solution was measured at 565 nm. For assessing the potential inhibitory activity of graded concentrations of elements of moth extracts. 100 to 400 µl extract was pre incubated with 300 µl enzyme solution at 37 ˚C for 30 min. Acarbose solution was used as a positive control. Along with raw and sprouts samples similar treatment given to only starch, only Enzyme and Substrate (i.e. enzyme and starch). The results were expressed as % inhibition of enzyme activity and calculated with the following equation:

% inhibition of α– amylase activity+ $(A_{ex}- A_e)/(A_s- A_e)$ *100, where A_{ex} is the absorbance of extract, A_e is the absorbance of the enzyme and$A_s$is the absorbance of the starch.

F. Alpha – Glucosidase Inhibition capacity:

The yeast α– glucosidase was dissolved in 100 mM phosphate buffer - pH 6.8 and was used as an enzyme extract. Moth extracts were used in the concentration ranging from 75 – 150 µl. Different concentrations of moth extracts were mixed with 0.1 M phosphate buffer pH 6.8 at 37 °C for 10 minutes. 5 µl. of P-Nitrophenyl-α-D-glucopyranoside (pNPG) was used as the substrate and incubated for 75 minutes at 37 °C. It hydrolysed by α - glycosidase and release p-nitro phenol. To stop the reaction 0.1 M Sodium carbonate(0.4 ml) was added to the mixture and the colour intensity measure at 405 nm [19, 20]. The control samples were prepared without moth extracts. The glucosidase inhibitory activity was expressed as percentage inhibition.
The % inhibition was calculated according to the formula: [2]

Inhibition % $=(A_{control-} A_{sample}) A_{control} \times 100$

STATISTICAL ANALYSIS

One way analysis of variance (ANOVA) was conducted, and the difference was determined by Duncan's post hoc test considering ($P \leq 0.05$)as the significant level of difference. Pearson's coefficients (r) were also calculated to establish the relationship among various parameters. SPSS version 20 was use to carry out statistical calculations.

RESULTS AND DISCUSSION

In this study, the effect of varying concentration of ascorbic acid elicitation on antioxidants and α-amylase and α- glucosidase inhibition of Moth beans was studied.The ascorbic acid elicitor is a powerful antioxidant and is synthesized in plant cell. It is also giving good response against both biotic and abiotic stress.
The total phenolic (TP) content was 22.16 mg% in raw moth beans and was increased significantly ($p \leq 0.05$) when moth beans were germinated with(T-3 to T-6) or without (T-2) ascorbic acid elicitation (table-1). The increase in TP in control germinated moth beans (T-2) was approximately 4 times and ranged from 4.5 to 6 times in T-3 to T-6. Varying concentration of ascorbic acid had a positive and linear effect on the TP in moth beans germinated with ascorbic acid elicitation. From there results, it can be said that ascorbic acid does induced a stress like stimuli, which increase TP in moth beans. The phenolic content in germinated lentils was increase significantly in response of ascorbic acid [21]. Previous studies [8, 21,23] suggested that ascorbic acid stimulate the PPP in

legumeas it related to deNOVOsynthesis of phenolic compounds and suggested that ascorbic acid stimulate the PPP and phenyl propanol pathways in legumes.Our findings are in accordance to previous studies of sprouted peas, favabeans and kidney beans [8, 21, 23]

Phenols and flavonoidsare an excellent antioxidant and prevent the oxidative disease [24].Flavonoids protects against oxidative stress by scavenging free radical or chelating process. Some findings coincides that during germination some secondary plant metabolites such as anthocyanin and flavonoids might be produce due to biochemical metabolism [8].

The Flavonoid present in moth beans is calculated by using a standard curve prepared with Rutin. Significantly higher ($p \leq 0.05$) flavonoid content was found in moth beans germinated with ascorbic acid i.e. T-6 and lowest value was obtained in raw i.e. 56.49 mg%. The results showed the positive and linear effect on flavonoid content in moth beans germinated with ascorbic acid. Kwan Kim [25] reported that the amount of flavonoid found in the mungsprouts lower than the moth beans compared to the raw moth beans.

Proline is found in higher amount in plant cell in response of various stress to plant. It is synthesized from glutamate [26]. This higher amount of proline in cell is basically due to deNOVO synthesis [27]. Proline can also provide stability to cellular components and act free radical scavenger [28]. Studies [26, 24] reported that stimulation of PPP increase proline content. The proline content was increased significantly ($P \leq 0.05$) in T-2 and T-3 to T-6 compared to raw moth beans (T-1). The ascorbic acid did not show the elicitation effect on Proline. In this study germination had a significant impact on Proline.

The total antioxidant capacity (TAC) is a total response of antioxidant compound to neutralize the free radicals in a system. There are many *In vitro* methods developed to measure TAC in plant foods with different principles. In this study FRAP, DPPH-RSA and ABTS-RSA were used to measure TAC of control and germinated moth beans. As many studies reported a positive and significant correlation among this three different *in vitro* total antioxidant capacity method.[8,22]

FRAP assay measures the antioxidant effect of substance in the reaction medium and it shows the total antioxidant power. The process is based on the ability of the samples to reduce $Fe^{3+}$to Fe^{2+} ions. The reducing power of the germinated samples was found higher than the raw. As germinated samples contain higher antioxidants which causes a higher reducing power [23].The FRAP was increased in germinated moth beans (T-2) and germinated moth beans with elicitation (T-3 to T-6) compared to T–1. The elicitation of moth beans with ascorbic acid showed a significant effect on FRAP. The FRAP content was increased approximately 8.5 to 9 times in T-2 to T-6 than the raw moth beans.

DPPH (2,2- Diphenyl – 1- picrylhydrazyl), is a stable organic free radical and but the absorption disappear due to reduction by an antioxidant. Formation of non –radical form (DPPH- H) is occurs due to the presence of hydrogen donating antioxidant compound and reduction of methanolic DPPH solution. Maximum adsorption of DPPH occurs at 517nm by accepting an electron[22].The methanolic extract of moth beans could contain some substances which are electron donors which convert free radicals to more stable product and stop the radical chain reactions.

ABTS radical cation was produced in the stable form using potassium persulphate and has been compared with standard Trolox. The moth beans extract was added to the reaction medium and the antioxidant power was measured by studying decolourization.Raw and germinated moth beans without ascorbic acid elicitation showed the similar ABTS–RSA. The T-3 to T–6 showed a significantly higher ABTS- RSA compared to T-1. There was a significant increase in ABTS-RSA when moth beans were germinated with ascorbic acid elicitation. There was no any linear response of ascorbic acid concentration on ABTS-RSA.

The results of FRAP, DPPH-RSA and ABTS-RSA are graphically presented in Figure.1. The increased total antioxidant in response of ascorbic acid elicitation is probably due to stimulation of PPP in legume [23].As mentioned earlier ascorbic acid is a potential antioxidant which might be a second possible cause of increasedTAC in germinated moth beans. Antioxidant activity was increased in dark

germinated favabeans sprouts in response of ascorbic acid eliciation and correlated the increased TAC with increased SOD and catalase activity. Various studies [19, 23, 24]reported increase in TAC in lentils, Fava beans, African yam beans and other legumes. Randhir et. al.[24] reported that the antioxidant activity was high in fava beans elicited with ascorbic acid due to increase in L – DOPA and phenolics content which might be contribute to increase the TAC.

Recently, dietary management has gain importance to control blood glucose levelabove 180 mg / dlin diabetics is considered as risk for developing secondary complications. The food rich in α- amylase and α- glucosidase inhibitors are known as hypoglycemic foods. They both are key enzymes to manage hyper glycemia[27].α- amylase inhibitors are starch blockers, which can bind with the reactive sites of amylase enzyme and reduce blood sugar level by altering its catalytic activity.The inhibitory effect of α-amylase and α- glucosidase in moth beans sprouts are graphically presented in Figure.2

The extract of moth beans (T-6) showed highest inhibitory activity Whereas T-1 showed 12.41 % of inhibition. There was no effect of ascorbic acid elicitation on α-amylase inhibition in moth beans.There results revealed that germination had a good impact on α- amylase inhibition and methanolic extract showed a comparable results with acarbose.

The α-glucosidase inhibition in raw moth beans was 95.2 %. Germination with (T-3 to T-6) and without (T-2) ascorbic acid elicitation did not showed any significant effect on α- glucosidase inhibition (table-2). The presence of phenolics and Flavonoids in the germinated legumes showed inhibitory effect on α- amylase and α-glucosidase activity.

In this study Pearson's correlation was studied to evaluate a relationship between various biochemical parameters. The results obtained are shown in table 2. The total phenolics had a positive and a significant correlation with Flavonoid, Proline Total antioxidant capacity and alpha amylase inhibition. It had a negative and significant correlation with alpha glucosidase inhibition. The proline had a similar trend of relation with Total phenol, Flavonoid, Alpha amylase, Total anti-oxidant capacity and alpha glucosidase. The correlation between three different methods of Total anti-oxidant capacity was found to be positive and significant.

CONCLUSION:

From the results of this study, the application of moth bean seed elicitation with ascorbic acid had a significant advantage to improve phenolic bioactive compounds which have linked with total antioxidant capacity and hypoglycaemic function. Therefore moth bean sprouts can be used in daily diet or for formulating food for diabetic populations.

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Table: 1 The total phenolics, Flavonoid and a Proline content of the raw, and germinated moth beans with ascorbic acid elicitor

Treatments	Total Phenols	Flavonoids	Proline
T-1	22.16 ± 0.98[a]	56.49 ± 4.00[a]	20.0 ± 0.44[a]
T-2	84.09± 1.94[b]	116.18 ± 1.60[b]	117.02 ± 1.05[d]
T-3	102.9 3 ± 5.02[c]	119.79 ± 8.00[b]	121.30 ± 6.99 [d]
T-4	114.7 ± 5.38[d]	135.24 ± 2.90[b]	85.57 ± 1.45 [b]
T-5	120.4 ± 4.25[d]	144.23 ± 2.92 [d]	97.03 ± 2.11 [c]
T-6	135.3 ± 7.34[e]	158.64 ± 4.12[e]	115.24 ± 3.06[d]
F- Value	297.91	260.93	526.81

Values are mean of ± S.D. of four observations. Mean value of different superscripts within a column are significantly different from each other ($p \leq 0.05$). GAE-Gallic acid equivalent, TE- Trolox – equivalent.

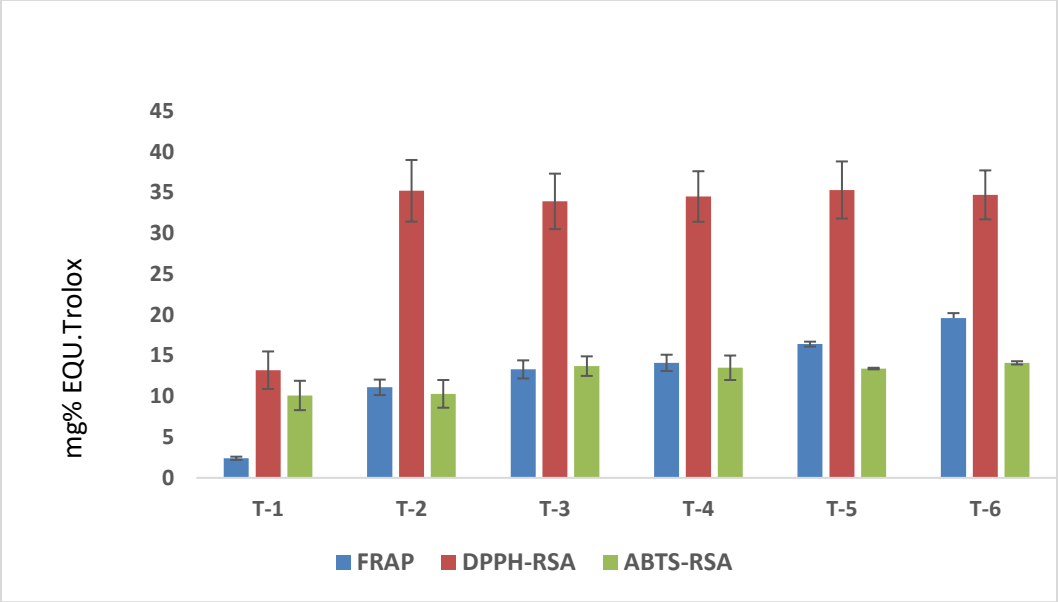

Fig.1 FRAP, DPPH –RSAand ABTS -RSA content of the Raw and germinated Mothbeans

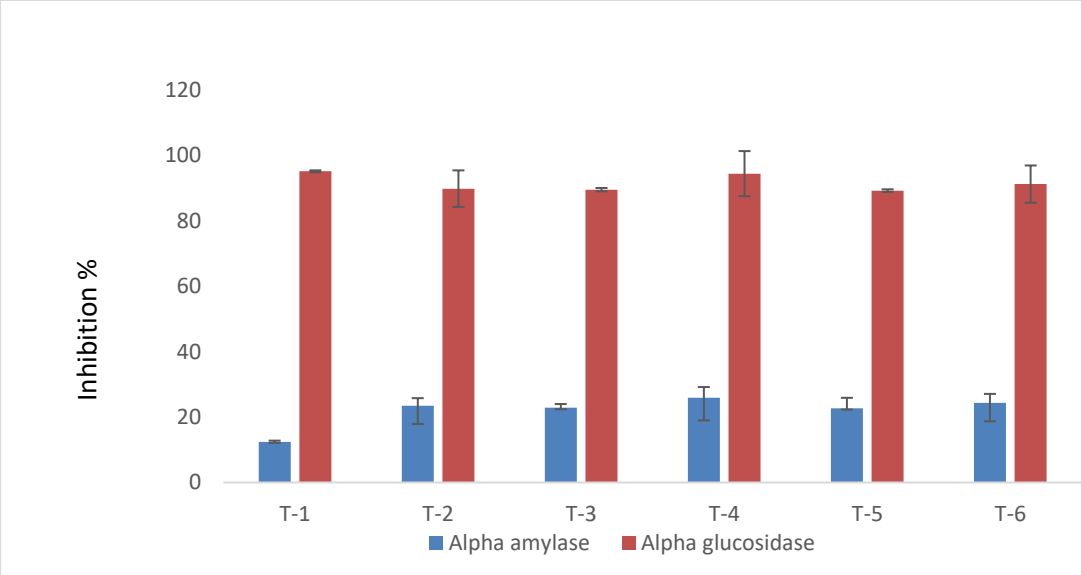

Figure: 2 Inhibitory effects of α- amylase and α- glucosidase in moth beans sprouts

Table:2 Pearson correlation among various parameters

	Total Phenolic	Flavonoids	FRAP	DPPH-RSA	ABTS-RSA	Proline	α-amylase inhibition	α- glucosidase inhibition
Total Phenolic	1	.981**	.969**	.873**	.687**	.785**	.772	.239**
Flavonoids	.981**	1	.982**	.845**	.651**	.787**	.796**	-.264
FRAP	.969**	.982**	1	.786**	.717**	.790**	.794**	-.310**
DPPH-RSA	.873**	.845**	.786**	1	.368*	.861**	.695**	-.197**
ABTS-RSA	.687**	.651**	.717**	.368*	1	.468*	583**	-.317**
Proline	.785**	.787**	.790**	.861*	.468*	1	.776**	-.423**
α- amylase inhibition	.772**	.796**	.794**	.695*	.583**	.776**	1	-.432**
α-glucosidase inhibition	-.239	-.264	-.310	-.197	-.317	-.423*	-.432	1

**correlation significant at the 0.01 level (1- tailed)
*Correlation is significant at the 0.05 level (1- tailed)

PRAJÑĀ - **Journal of Pure and Applied Sciences Vol. 27-28: 62 - 69 (2019-20)**
ISSN 0975 - 2595

IMPACT OF HIGH PROTEIN LADOO SUPPLEMENTATION ON THE NUTRITIONAL STATUS OF MALNOURISHED CHILDREN

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ABSTRACT

Malnutrition continues to be a major health burden in developing countries especially in children under five years. India is still habitat to the world's highest demographics of children suffering from malnutrition. Malnourished children are best treated with specially formulated foods known as ready-to-use foods (RUFs) which can balance the diet to provide additional nutrients. In this study, High protein ladoo (HPL) containing soybean, cowpea, rice flour, gingelly seeds, SMP, jaggery and ghee were prepared. Nutritionally, 100g of ladoo contained 510kcal energy, 41.57g% carbohydrate, 17.96 g% protein, 30. 22g% fat and 88.3mg% calcium. The developed ladoo (70g, 2 in no.) providing 357.07kcal and 12.57g protein was supplemented to moderately acute malnourished children enrolled in the experimental group (n=21) for 60 days. The control group (n=11) did not receive any nutritional supplement. A significant improvement was observed in anthropometric measurements such as weight, height, mid upper arm circumference (MUAC) and body mass index (BMI) in the experimental group. WHZ and WAZ also showed significant improvement whereas HAZ did not show any significant change in the experimental group. At the end of supplementation 80.95% of the moderately wasted children (WHZ) and 76.19% of the moderately underweight (WAZ) children improved from the moderately malnourished category to the mildly malnourished category.

Keywords: High protein ladoo, supplementary feeding, nutritional status.

INTRODUCTION

Malnutrition continues to be a major health burden in developing countries especially in children below the age of five years. More than 200 million children in developing countries do not reach their full developmental potential because of poverty, poor nutrition and inadequate care [1] and this results in an increased prevalence of moderate acute malnutrition (MAM) and poor cognitive development [2]. Moderate acute malnutrition (MAM) is defined as weight for height between -3 SD and -2 SD below the median weight for height of the WHO child growth standards [3]. Even with the growing per capita income, sustained economic growth and increased agricultural production, malnutrition continues to beat India in multiple forms. India is still habitat to the world's highest demographics of children suffering from malnutrition [4]. According to NFHS-4 data, 38.4% of under-five children are found to be stunted, 21% wasted and 35.8 % underweight in India whereas in Gujarat 39.3% of children under the age of five are underweight, 26.4% are wasted and 38.5% are stunted [5].

MAM children are best treated with specially formulated foods which can balance the diet to provide additional nutrients [6]. During the 1960s, fortified blended cereal based foods like corn-soy blend as well as other legumes blended with soy were introduced for improvement in the nutrient content and to increase the protein quality [7]. A relatively newer category of specially formulated foods known as ready-to-use therapeutic foods (RUTFs) or ready-to-use supplementary foods (RUSFs) have been introduced by WHO (World Health Organization), recently. These ready-to-use foods (RUFs) are energy-dense, high fat, ready-to-eat pastes often made from a combination of peanuts, milk powder and/or soy, sugar, oil and micronutrients. WHO advocates the use of optimal locally available nutrient-dense foods to prevent children from becoming severely malnourished [6].

In the present study High protein ladoo containing soybean, cowpea, rice flour, gingelly seeds, SMP, jaggery and ghee were prepared. Soybean is referred to as the meat for vegetarians due to its higher quantity and quality of protein. Along with a high quality of amino acid profile it also contains carbohydrate, fat, vitamins and minerals such as folic acid, calcium, potassium and iron **[8]**. Potential health benefits of soy may be limited due to the presence of antinutritional factors, including trypsin inhibitors. But processing techniques like

germination, soaking, dehulling and roasting reduces antinutritional factors from soybeans **[9]**. Several studies suggest the use of soybean as a supplementary food to combat malnutrition in children **[10, 11]**. Cowpea is an underutilized crop with a good protein quality. Rice flour and skimmed milk powder (SMP) contain protein of high quality while gingelly seeds and soya provide omega 3 fatty acids essential for improved growth.

In the present study the impact of high protein ladoo supplementation on the nutritional status of malnourished children (3-5 yrs old) was studied. The specific objective of the study was

- To study the effect of High protein ladoo supplementation on moderately malnourished children (3-5 years old) with respect to growth parameters such as weight for age (WAZ), height for age (HAZ) and WHZ (weight for height).

METHODOLOGY

The methodology is discussed under the following heads:

A. Development of High protein ladoo
B. Nutrient analysis of ladoo
C. Supplementation of ladoo
D. Data analysis

A. Development of High protein ladoo:

Different ingredients such as soybeans, cowpea dal, rice flour, gingelly seeds, skimmed milk powder (SMP), jaggery and ghee (Amul) were procured from the local market of Anand. Soya beans were soaked for 12 hours followed by dehulling, pressure cooking (15 min), sun drying (48 hours) and roasting (15 min). Roasted soya beans were ground to make flour. Cowpea dal was roasted (15 min) and ground to flour. Rice was soaked (12 hrs), sun dried, roasted (15 min) and flour was prepared. Gingelly seeds were cleaned, roasted (15 min) and ground. All the ingredients were mixed in the specific proportion to provide an essential amino acid profile close to the reference protein (egg) by NIN, 2017 **(Fig.1)** and ladoo was prepared.

B. Nutrient analysis of ladoo:

Proximate composition of the developed High protein ladoo was studied. Parameters analyzed were moisture (AOAC, 2000) **[12]**, ash (AOAC, 2000) **[12]**, fat (Sohxlet Sosplus- scs 03) and protein (Pelican Kelplus Supra lxva Instrument). Minerals (calcium, iron, phosphorus, copper and zinc) were analyzed using Inductive Coupled Plasma- Optical Emission Spectroscopy [Perkin Elmer: Avio 200 (ICP-OES)].

C. Supplementation of ladoo:

- Sample size: A sample size of 32 was derived using Open Epi software considering 95% significance level at a power of 80% and assuming that 60 % children will improve in their body weight after supplementation.
- Selection of subjects: Written permission was obtained for conducting the supplementation study from two purposively selected balwadi centers of the lower middle income group situated at V. V. Nagar in Anand district. Selection of balwadi centers was based on compliance obtained from balwadi centers and proximity. All the children attending either of the two selected balwali (n=2) were evaluated for their nutritional status based on anthropometric measurements.

  Based on cut-offs given by WHO, children were classified into normal, mild or moderately malnourished (underweight/ stunted/ wasted). Malnourished children were then selected based on a Z score between -2 and -3 SD. A total of 32 MAM (WHZ <-2 and ≥-3 without oedema, WAZ <-2 and ≥-3) children aged between 3-5 years were purposively enrolled in the study. The children were divided into control (n=11) and experiment (n=21) groups.
- Exclusion criterion: Children suffering from any infectious disease at the time of start of the study were not included in the study.
- Written consent:All parents of the enrolled MAM children were explained in detail about the objective and the methodology of the study. All parents were informed about the ingredients, the processing of the ingredients and the preparation method of the ladoo before the clinical trial. Before the start of the study, ladoo was distributed to the mothers of the subjects in order to help them decide about joining the study. Based

on the willingness to take part in the study written consent was taken from the parents.

- Feeding the ladoo: The supplementation study was conducted for a period of 60 days between December to February, 2019. Ladoo was prepared freshly and 70 grams (2 in number) were packed in polyethene bags and distributed to the MAM children of the experimental group at the balwadi centers. 70 g was decided based on the energy and protein levels supplied by supplementary feeds. Subjects were instructed to finish consuming the ladoo during the same day. Children from control group did not receive any food supplement.
- Parameters studied:
  - ✓ Height (cm), weight (kg) and mid arm circumference (MUAC) (cm) were recorded as per standard methods.
  - ✓ Body mass index (BMI) and Z score for weight for height (WHZ - wasting), weight for age (WAZ - Underweight) and height for age (HAZ - stunting) were calculated using WHO Anthro software (v 3.2.2).
- ✓ Weight gain was calculated using the formula given below **[13]** where, W1 is the initial weight in kg and W2 is the weight in kg on the last day of measurement.
- ✓ Weight gain in g/kg/day

$$= \frac{(W2 - W1)x\ 1000}{(W1\ x\ Number\ of\ days\ from\ W1\ to\ W2)}$$

D. Data analysis: Data was analyzed using SPSS version 19.0 (Statistical Package for Social Sciences). Comparison of means was performed using paired t- test. A p- value of $\leq$ 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

A. Nutrient analysis:

High protein ladoo was developed from soybean, cowpea, rice, SMP, Jaggery and ghee as a RUF for the treatment of malnourished children. Proximate composition of the high protein ladoo is depicted in **Table 1**.The developed ladoo provided 510 kcal of energy, 18 g protein, 30 g fat and 88 mg of calcium per 100 g. while 70 g is the quantity of ladoo used for feeding which provided 357 kcal energy, 12.57 g of protein, 21.15 g fat and 61.81mg of calcium per day. The cost of the ladoo was 20/- for 70 g of ladoo.

B. Screening of subjects:

For the supplementation study, all the children from the two selected balwadi centers were evaluated for their nutritional status. About 100 children were included in the screening phase. Based on their Z score for WAZ, HAZ and WHZ children were categorized. Confirmation of the date of birth resulted in 21% of the children being excluded during the screening. The prevalence of WAZ, HAZ and WHZ in the children included for screening is presented in **Fig 2.** The prevalence of WHZ was found to be high (38%) compared to WAZ (36.7 %) and HAZ (27.8 %) among the children included in the screening.

C. Effect of supplementation

A total of 33 children were enrolled in the study. At the end of the study one child suffering from infection dropped out. Therefore, 32 children (40.6% boys, 59.4% girls) remained for follow up, of whom 21 received ladoo supplementation and 11 did not receive any supplement. Anthropometric changes after 60 days of supplementation in both the groups are presented in **Table. 2**. Percentage change in all the anthropometric parameters are presented in **Fig 3(a) & (b)**. It showed that the average weight of control and experimental group was 11.72 ± 0.255 kg and 11.82 ± 0.256 kg, respectively, before the supplementation of high protein ladoo which was nearly similar in both the groups. After 60 days of supplementation to the experimental group, it increased significantly ($p<0.01$) in the control and the experimental group by 3.24 % and 10.40%, respectively. A similar trend was observed for height also. After supplementation, height increased significantly by 1.45 % and 1.95 % in control and experimental groups, respectively. The average value of MUAC for the control group was 13.85 ± 0.208 cm and 13.97 ± 0.185 cm before and after supplementation, respectively which showed no significant ($p=0.552$) difference whereas MUAC increased significantly ($p=0.003$) by 2.91% in the experimental group after supplementation. There was no significant

difference in the BMI of the children in the control group after supplementation. In the experimental group BMI was found to be 11.42 ± 0.926 to 13.51 ± 0.149 kg/m^2 before and after, respectively. A significant ($p<0.05$) increase was observed in the BMI of experimental group after ladoo supplementation by 18.3 %.

Studies conducted by several researchers showed an increase in the anthropometric measurements after soy ladoo supplementation to preschool children for six months **[14]**. Sharma et al (2018) also reported a % change in height by 0.90%, weight by 9.06% and BMI by 8.13% in the group supplemented with soy poha ladoo for 90 days **[15].** In the present study the weight gain of the children in the supplementation group was significantly higher due to the nutrient content of the ladoo. The developed ladoo was energy dense and high in protein quantity and quality. All the preprocessing treatments such as soaking and dehulling given to the ingredients used in the preparation of ladoo has also promoted significant reduction in the antinutritional factors. Soaking reduces phytate content along with other antinutritional factors such as saponins, trypsin inhibitors and polyphenols from the soybean. Heat processing treatment such as roasting increases digestibility of proteins, carbohydrates and other nutrients and enhances the flavor. Additionally cereal and pulse combination in the preparation of the ladoo has provided a complete protein essential for proper growth and development. All the ingredients used in the preparation and the amounts were fixed in a proportion such that all the essential nutrients especially amino acids were provided in the required amount **[16]**.

Z-score, also known as Standard Deviation (SD) score is the measure of dispersion/relative deviance of the data from the mean/median value i.e. measure of the distance between the child's value and value of the reference population. Nutritional status is well predicted by calculating Z-score for weight-for-height, height-for-age and weight-for-age. As summarized in Table 2, the mean WAZ and WHZ score improved significantly ($p<0.01$) in the experimental group after supplementation of ladoo for 60 days whereas no significant difference was observed in HAZ after supplementation. As far as control group is concerned no significant difference was seen in WAZ, HAZ and WHZ before and after supplementation. The mean weight gain for the control group was 0.53 ± 0.114 g/kg/day and in the experimental group it was observed to be 1.74 ± 0.154 g/kg/d after supplementation which showed a highly significant ($p=0.004$) difference. A randomized controlled trial also reported weight gain velocity to be 2.71 g/kg/day in moderately acute malnourished children treated with ready-to-use supplementary food in Camerron **[17]**. In the present study, at the end of the supplementation period 80.95% of the moderately wasted children (WHZ) recovered and shifted to the yellow zone of mild malnutrition whereas 76.19% of the moderately underweight (WAZ) children also improved from MAM. However, in the study done by Gabriel (2015) **[17]** recovery rate in the group of MAM children supplemented with RUSF based on soy and corn flours, peanut paste, sugar, soy oil and premix containing minerals and vitamins was reported to be 73%.

CONCLUSION

Based on the above findings, it can be concluded that the developed high protein ladoo as a ready-to-use supplementary food had a significant effect on the weight, height, MUAC, BMI, WAZ and WHZ of MAM children in the experimental group. Supplementary foods made from locally available ingredients combined with house hold processing techniques have shown to be highly effective in this study. Thus the use of locally available and underutilized ingredients needs to be promoted for the production of supplementary foods in the prevention and treatment of malnourished children.

Children are the future of the nation and the nutritional status of under five children is an important indicator of the overall development of the community and of the nation. Healthy children will develop into healthy adults hence the developed supplementary food should be advocated in government feeding programs.

ACKNOWLEDGEMENT

This study was supported by a grant from UGC Meritorious BSR Fellowship (Sanction No.F.25 1/2014-15(BSR)/F.7-408/2012), Government of India, New Delhi. I am also thankful to the president of balwadi centers and parents of the children for their cooperation.

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Table 1: Proximate composition of High protein ladoo

Nutrients	100g	70 g (2 in number)
Energy (Kcal)*	510.10	357.07
Carbohydrate (g)*	41.57	29.09
Protein (g)	17.96	12.57
Fat (g)	30.22	21.15
Moisture (g)	1.7	1.19
Ash (g)	3.80	2.66
Calcium (mg)	88.31	61.81
Iron (mg)	0.73	0.51
Phosphorus (mg)	81.83	57.27
Copper (mg)	0.10	0.07
Zinc (mg)	0.56	0.39

*calculated values (NIN, 2017) **[18]**

Table-2 : Anthropometric changes after supplementation of High protein ladoo.

Anthropometric parameters	Control (n=11)		Experiment (n=21)	
	Before	After	Before	After
Weight (kg)	11.72 ± 0.255	12.10** ± 0.257	11.82 ± 0.256	13.05** ± 0.265
Height (cm)	93.90 ± 1.368	95.27** ±1.395	96.49 ± 1.14	98.38** ± 1.134
MUAC(cm)	13.85 ± 0.208	13.97^{NS} ± 0.185	14.05 ± 0.149	14.46** ± 0.147
BMI(kg/m^2)	13.30 ± 0.151	13.35 ^{NS} ± 0.155	11.42 ± 0.926	13.51* ± 0.149
Weight for age –Z score (WAZ)	-2.40 ± 0.175	-2.35 ^{NS} ±0.178	-2.27 ± 0.155	-1.78** ± 0.144
Height for age-Z score (HAZ)	-1.92 ± 0.334	-1.961 ^{NS} ± 0.321	-1.43 ± 0.232	-1.31 ^{NS} ± 0.212
Weight for height-Z score(WHZ)	-1.92 ± 0.126	-1.78 ^{NS} ± 0.130	-2.35 ± 0.124	-1.54** ± 0.115

Values are the mean ± SEM.
*and ** indicate significant difference at $p \leq 0.05$ and $p \leq 0.01$, respectively.
NS: Non significant

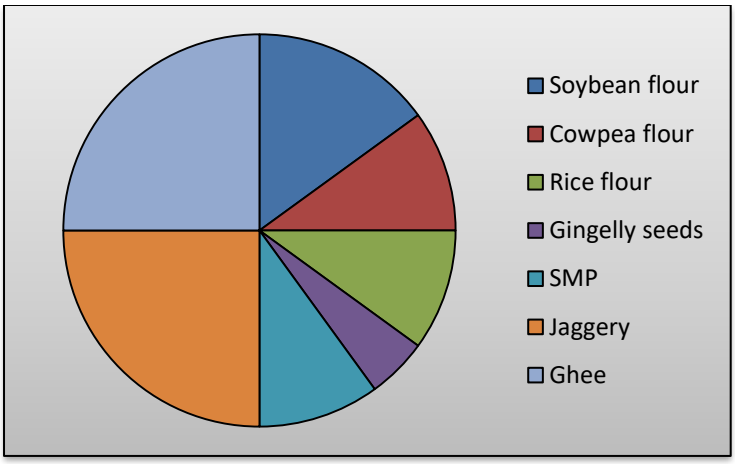

Fig 1 Composition of High protein ladoo

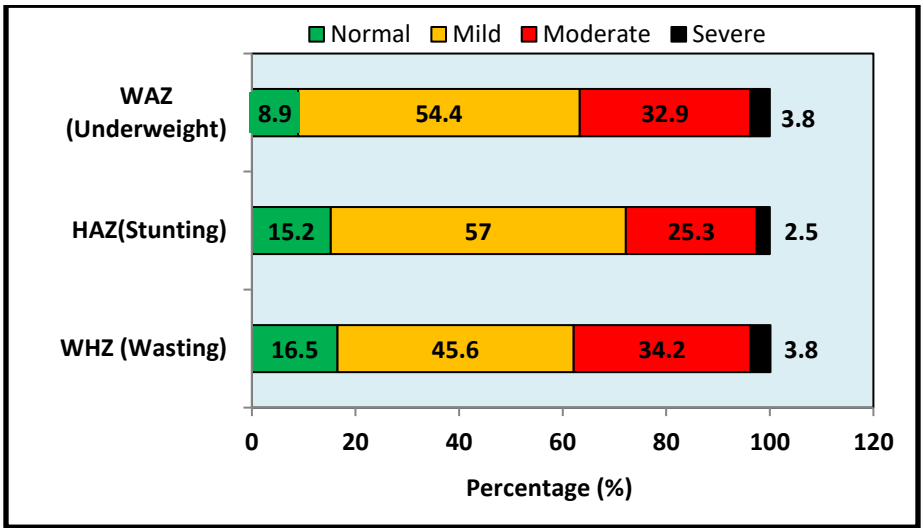

Fig 2: Prevalence of WAZ, WHZ and HAZ among the children (n=79) included in the screening

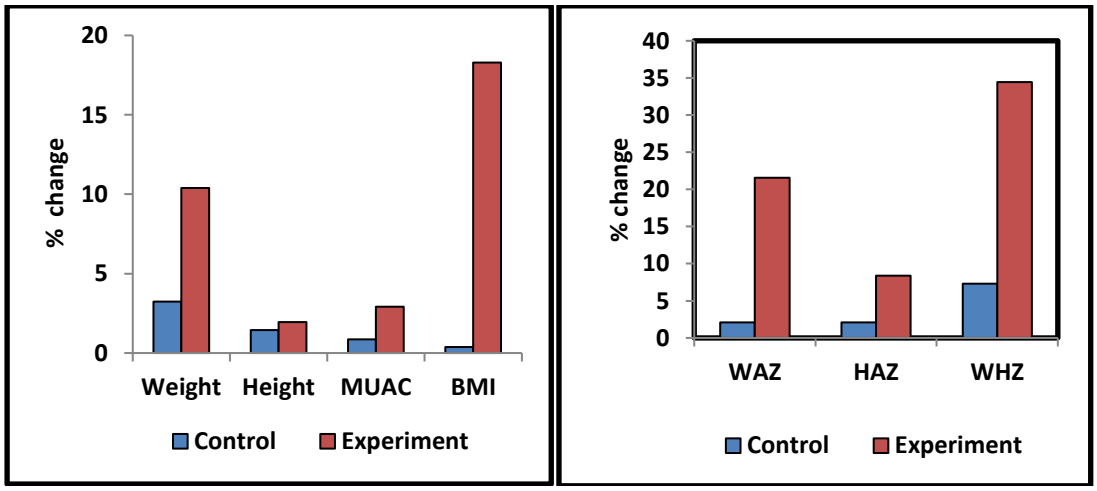

Fig 3(a) & (b): Percent (%) change in anthropometric parameters

PRAJÑĀ - **Journal of Pure and Applied Sciences Vol. 27-28: 70 - 72 (2019-20)**
ISSN 0975 - 2595

Shell-Crossing and Shell-Focusing Singularity in Spherically Symmetric Spacetime

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ABSTRACT

Starting from Schwarzschild exterior solution singularity has attracted researchers. There are conjectures (like cosmic censorship conjecture) which suggest that the singularities are to be covered by event horizon. However these are examples of naked singularities, thus it is always interesting to discuss singularities in this context.

Spacetime singularities are classified as shell-crossing and shell-focusing also; in this paper we consider spherically symmetric spacetimes filled with dust and perfect fluid. We find that in these cases both type singularities occur simultaneously.

Keywords: Shell-crossing, Shell-focusing, Naked singularity

INTRODUCTION

The Einstein's theory of relativity prophecies that the end fate of collapsing matter which exhausted its threadbare nuclear fuel. If star is more massive than few solar masses it could undergo an endless gravitational collapse without earning any stable stage. When the star wiped out it's inter nuclear fuel which provided outward pressure against inward pull of gravitation filed

The key point of this analysis is to find out the behavior of spacetime singularity of collapsing matter cloud in which the radius of star converse to a small positive value. The result form gravitational collapse is highly dense region with strong gravity and physical quantity such as density and Kretschmann curvature scalar could blow-up. If the event horizon starts developing before such a collapse, the collapsing matter gets hidden within a horizon. As result of collapse black hole created. If the developments of event horizon gets late during the collapse then the final result is the naked singularity, which can send out information to observer from strong gravity region. The cosmic censorship conjecture showed that trapped surface gives spacetime singularity; such a singularity must always hide behind event horizon of gravity.

The gravitational collapse of massive cloud could it be inspected using Einstein field equations. Here the main point in the theory of collapsing matter cloud is that the creation of shell-crossing and shell-focusing singularities. The shell of matter implode in such a way that fast moving outer shell of matter overtake the inner shells and producing a weak singularity this is called shell-crossing singularity where density and curvature scalar $\mathrm{K} = R^{hijk}R_{hijk}$ blow-up.

On the other hand shell-focusing singularity occurring at center of spherically symmetric collapsing matter, this is genuine curvature singularity where Kretschmann curvature scalar assume unboundedly large value. Many times singularity is observed due to bad choice of coordinate. To examine a singularity due to coordinates is done by checking the Kretschmann curvature scalar. We consider shell-crossing as a weak singularity because the volume element along to geodesics are non-zero, we can remove shell-crossing singularity with suitable extension of spacetime.

Spherically Symmetric Spacetime

The final stage of gravitational collapsing matter we consider here idealization, spherically symmetry. The advantage is that it

can solve analytically to get exact solution. The first studies examining the dynamical collapsing matter cloud by Oppenheimer and Snyder [3], and Datt [9]. The naked nature of gravitational collapse of inhomogeneous dust cloud (zero pressure) has been studies in details by Datt [9]. Here we need investigating only the gravitational collapse with two real orthogonal eigenvectors of spherically symmetric metric. We take the matter field with weak energy condition. The energy density as measured by any observer is positive for any timelike vector V^i.

$$T_{ij}V^iV^j \geq 0 \qquad (1)$$

Shell-Crossing and Shell-Focusing Singularity

Here we consider perfect matter fluid, the idealized condition and in order to determine field equation and analyze shell-crossing and shell-focusing singularity. Such matter field, can be given as

$$T_{ij} = (\rho + p)V_iV_j - pg_{ij} \qquad (2)$$

For a spherically symmetric matter distribution, choose the co-ordinates t, r, θ, ϕ, the metric is written as,

$$ds^2 = -e^{2\nu}dt^2 + e^{2\psi}dr^2 + R^2d\Omega^2, \qquad (3)$$

where $d\Omega^2 = d\theta^2 + sin\theta\, d\phi^2$ and ν, ψ and R are function of t and r and the stress energy tensor T^{ij} as given by equation (2) has only diagonal component in co-moving coordinate system

$$T_{tt} = -\rho\,, T_{rr} = T_{\theta\theta} = T_{\phi\phi} = p\,, \qquad (4)$$
$$T_{ij} = 0, \quad for\ i \neq j$$

Here ρ is density and p is pressure which are eigenvalues of T^{ij}. Here the energy density as measured by any observer must be positive. The weak energy condition holds for matter with two eigenvectors provided,

$$\rho \geq 0, \rho + p \geq 0 \qquad (5)$$

The Einstein field equations for metric (3) gives,

$$p = \frac{1}{R^2}\begin{bmatrix} e^{-2\nu}\left(-\left(\dot{\nu} - \dot{\psi}\right)\left(\dot{R} - R\dot{\psi}\right) + \ddot{R} + R\ddot{\psi}\right) \\ -e^{-2\psi}\left((R' + R\nu')(\nu' - \psi') + R + \mathrm{R}\nu\right)\end{bmatrix} (6)$$

$$= \frac{1}{R^2}\begin{bmatrix} -e^{-2\psi}R'(R' + 2R\nu') + e^{-2\nu}(e^{2\nu} + \dot{R}^2 \\ -2R\dot{R}\nu + \dot{2}\ddot{R})\end{bmatrix} (7)$$

$$\rho = \frac{1}{R^2}[e^{-2\psi}(R'^2 - 2RR'\psi' + 2RR'') + e^{-2\nu}(e^{-2\nu} + \dot{R}^2 - 2R\dot{R}\dot{\psi})] \qquad (8)$$

$$0 = -2[\psi R' + R\nu' - R'] \qquad (9)$$

Now,

$$\rho = \frac{1}{R^2}[e^{-2\psi}(R'^2 - 2RR'\psi' + 2RR'') + e^{-2\nu}(e^{-2\nu} + \dot{R}^2 - 2R\dot{R}\dot{\psi})]$$

Using, $-2[\dot{\psi}R' + \dot{R}\nu' - \dot{R}'] = 0$,

$$\rho = \frac{\begin{matrix} e^{-\psi}(R'^2 + 2RR'\psi' - RR') \\ +e^{-2\nu}\left(\dot{R}^2 + \frac{2}{R'}\left(-R\dot{R}\nu' + R\dot{R}\dot{R}'\right)\right) + 1 \end{matrix}}{R^2} \qquad (10)$$

Or,

$$\rho = \frac{1}{R^2R'}\begin{bmatrix} R'\left\{1 + e^{-2\nu}\dot{R}^2 - e^{-2\psi}R'^2\right\} \\ +R\left\{2e^{-2\nu}\dot{R}\left(R'\dot{R}'R'' - \dot{R}\nu'\right) + 2e^{-2\psi}R'^2\psi'\right\}\end{bmatrix} \qquad (11)$$

$$\rho = \frac{-F'}{R^2R'} \qquad (12)$$

$$p = \frac{\dot{F}}{R^2\dot{R}} \qquad (13)$$

$$2R' = 2R'\frac{\dot{G}}{G} + \dot{R}\frac{H'}{H} \qquad (14)$$

$$\nu' = \frac{p'}{\rho + p} \qquad (15)$$

$$F = R(1 - G + H) \qquad (16)$$

Functions H and G are defined as,

$$H = e^{-2\nu(r,t)}\dot{R}^2$$

$$G = e^{-2\psi(r,t)}R'^2$$

Here the prime and dot denote partial derivative with respect to the parameters r and t respectively. $F = F(r,t)$ the Misner-Sharp mass function with $F \geq 0$. In above non-linear system we have five equation and six unknowns that is to say R, F, G, H and ρ and p. Here ρ is the positive density; p is pressure which is uniform throughout collapse. We are only affiliated with the gravitational collapse of perfect fluid. For the collapse condition,

$$\dot{R}(t,r) < 0$$

In general, R' may not be positive. The inner shells are more weak then outer shells.
For spherically symmetric continual collapse energy-mass density is given by.

$$\rho = \frac{F'}{R^2 R'}$$

This condition trivially leads to shell-crossing and shell-focusing, generally this shell-crossing as weak singularity with dust ball and perfect fluid. The volume elements along the geodesics are converse to non-zero so this is weak surface singularity.
The shell-crossing singularities were studied by Szekeres [4] and Lun [4] who investigated Newtonian spherically symmetric dust ball solution. In Tolman dust ball solution Hellaby [2] and lake [2](1995) define necessary and sufficient condition for shell-crossing.

CONCLUSION

The study of spherically symmetric collapse out here reveals that non-removable shell-crossing singularity occurs. Also, the singularity here is genuine shell-focusing curvature singularity. Here we considered perfect fluid as material content of spherical body. Our results match with the dust situation considered in Datt [9]. We hope that this method can be applied in more general situation.

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PRAJÑĀ - Journal of Pure and Applied Sciences Vol. 27-28: 73 - 81 (2019-20)
ISSN 0975 - 2595

Synthesis of adsorbent from agricultural waste to remove heavy metal ions from waste water

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ABSTRACT

Water pollution is a global concern and it is the high time that we realize the gravity of the situation. Removing pollutants from the water is most important today. Developing a cost effective and environmentally safe method is a challenging task for chemical engineers. The presence of heavy metal ions greatly influences the quality of water and the removal of this kind of pollutant is of prime importance. Using many methods by which adsorbents can be manufactured such as enhancement of porosity or activating the adsorbent by carbonization.

Key wards: Agricultural Waste, carbonization, Adsorption isotherms, Langmuir isotherm equation.

INTRODUCTION

1.1 Prelude

Saving water to save the planet and to make future of mankind safe is what we need now. With the growth of mankind, society, science, technology, our world is reaching to new high horizons but the cost which we are paying or will pay in near future is surely going to be too high. Among the consequences of this rapid growth is environmental disorder with a big pollution problem. Anthropogenic activities have caused a great harm to the quality of our lifeline, i.e. water. Because of fast depletion of freshwater resources, there seems to be a crisis of the same. Water pollution is a global concern and, it is the high time that we realize the gravity of the situation. Removing pollutants from the water is crying need of the hour and developing a cost effective and environmentally safe method to achieve the same is a challenging task for chemical engineers. After all, it is the future of mankind, which is at stake.

1.2 Background of Present Research

Now – a – days, a large amount of waste water having heavy metal ions is generated by many industries like textile, dye, battery, automobiles and so on [1]. The presence of heavy metal ions greatly influences the quality of water and the removal of this kind of pollutant is of prime importance. Owing to the hardness possessed by heavy metal ions they are difficult to treat with municipal waste treatment operations [2]. Even a small quantity of heavy metal ion causes high

undesirability. Moreover, the harmful effects caused by presence of heavy metal ions in water are aesthetically unpleasant [1]. They can have very harmful effects when exposed to human beings, animals, plants and many more. In addition to that, many heavy metal ions are considered to be toxic and even carcinogenic.

Few decades earlier presence of heavy metal ions in waters their effects and consequences were not given much importance. With the growing health concerns, it was in the 80s when people started paying much attention to the heavy metal ion waste that are present in water [3]. An indication to the magnitude of this problem can be inferred from the fact that almost all the waste water produced by the industries are directly discharged into aqueous effluents [4, 5]. With the increased stringent laws on industrial discharge, it has become very important to treat this waste water. Because of their detriment and large-scale distribution in the ecological environment, their separation and determination has become one of the important studies of environmental analysis. Of prime importance is the need for clear information on the safety related properties of the metal ions and the measure to be taken for lowering their exposure. If all these elements are seriously considered, then the technical use metal ions and the handling involved might be possible without much health danger.

1.3 Research Objectives

Several physical and chemical methods such as coagulation/flocculation treatment, oxidation methods, membrane filtration and adsorption have been reported to be investigated for the removal of heavy metal ions from industrial effluents [6-9]. Among the studied methods, removal of heavy metal ions from adsorption is found to be most competitive one because it does not need a high operating temperature and several other impurities can be removed simultaneously [1]. The versatility of adsorption is due to its high efficiency, economic feasibility and simplicity of design [10]. As there are various parameters to effect adsorption process such as charge, density and structural stability of the adsorbent so, in the thrust of a comprehensive study, we have selected two waste containing heavy metal ion of lead and other of copper respectively for this present study.

Several adsorbents made from various agricultural wastes such as corn husk, rice husk, groundnut shell, sugarcane bagasse have been used for the purpose of checking the removal of copper and lead heavy metal ions from water. One of the conventional and most studied adsorbent is Activated Carbon (AC). The reasons for this popularity are its high adsorption capacity, high surface area and microporous structure but the cost of activated carbon or for the process of activation is very high [2].

Thus, our objective is to study the effects of adsorbents made from agricultural waste like rice and corn husk in removal of heavy metal ions i.e. lead and copper ions without activation and also to remove the heavy metal ions effectively from

the waste water and also to find the adsorption isotherms.

MATERIALS AND METHODS:

2.1 Materials

2.1.1 Required Chemicals

1. 0.01M zinc sulfate solution
2. 0.01M EDTA
3. pH 10 Buffer solution
4. 0.01M Lead acetate solution
5. Distilled water
6. EBT
7. 0.01M Copper sulfate solution

2.1.2 Raw material

Corn husk, Rice husk

2.1.3 Equipment used

1. Gasifier
2. Sieve shaker
3. 250 ml conical flask
4. Magnetic stirrer
5. 1000 ml beaker
6. UV Spectrometer

2.2 Method of Synthesis

There are many methods by which adsorbents can be manufactured such as enhancement of porosity or activating the adsorbent by carbonization. Activated carbon can be produced by increment in porosity and also by carbonization. Some adsorbents can be produced from different agricultural waste by gasifying the agricultural waste.

2.2.1 Production of adsorbent from corn husk

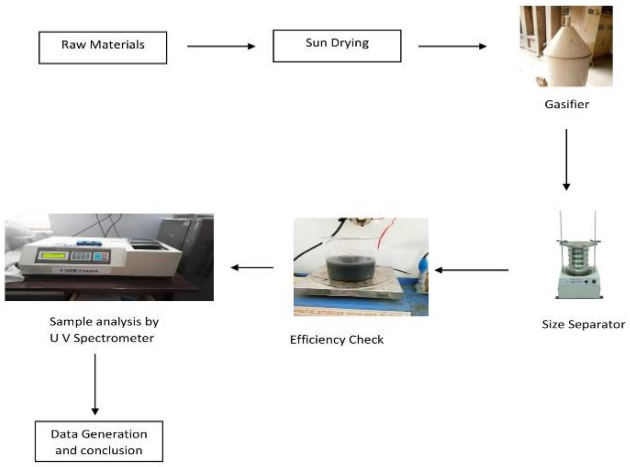

Fig 2.1: Experimental procedure

Initially the raw material (agricultural Waste) is dried in the sun, then the dried waste is burned in gasifier and the ash from the gasifier is collected. This ash works as adsorbent. Then with the help of sieve shaker the ash (adsorbent) is maintained in proper mesh size. This adsorbent can be used directly to remove heavy metal ions without activation. The adsorbent is added in the reactor having waste containing heavy metal ions. By giving the proper residence time the heavy metal is adsorbed on the adsorbent. The percentage removal can be measured from the UV spectrometer and then the data are generated. From the data isotherms are plotted.

RESULTS AND DISCUSSION:

Data's for removal of lead and copper ions using rice and corn husk were generated based on that isotherms are plotted and the satisfactory conclusion is obtained from the generated results.

3.1 Experimental Data for Copper and Lead Using Rice Husk

3.1.1 Experimental data for copper

Table 3.1: Data for copper using rice husk

Time (min)	Concentration (mg/lit)	% Removal
0	30	0
5	12.954	56.82
10	10.809	63.97
15	8.733	70.89
20	7.407	75.31
30	6.171	79.43
40	5.307	82.31
50	4.716	84.28
70	4.089	86.37
90	3.657	87.81

3.1.2 Experimental data for lead

Table 3.2: Data for lead using rice husk

Time (min)	Concentration (mg/lit)	% Removal
0	30	0
5	7.926	73.58
10	3.987	86.71
15	2.541	91.53
20	2.292	92.36
30	2.076	93.08
40	1.875	93.75
50	1.701	94.33
70	1.611	94.63
90	1.473	95.09

3.1.3 Results for lead and copper using rice husk

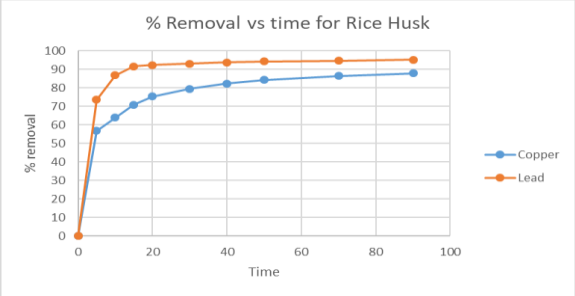

Fig 3.1: Graph of %Removal vs Time for rice husk

From the above graph we can say that adsorbent made from rice husk removes lead more effectively compared to copper.

3.2 Experimental Data for Copper and Lead Using Corn Husk

3.2.1 Experimental data for copper

Table 3.3: Data for copper using corn husk

Time (min)	Concentration (mg/lit)	% Removal
0	30	0
5	9.654	67.82
10	6.915	76.95
15	5.442	81.86
20	4.446	85.18
30	3.891	87.03
40	3.426	88.58
50	2.961	90.13
70	2.433	91.89
90	2.121	92.93

3.2.2 Experimental data for lead

Table 3.4: Data for lead using corn husk

Time (min)	Concentration (mg/lit)	% Removal
0	30	0
5	10.815	63.95
10	8.154	72.82
15	6.321	78.93
20	5.418	81.94
30	4.731	84.23
40	4.176	86.08
50	3.621	87.93
70	3.291	89.03
90	3.081	89.73

3.2.3 Results for lead and copper using corn husk

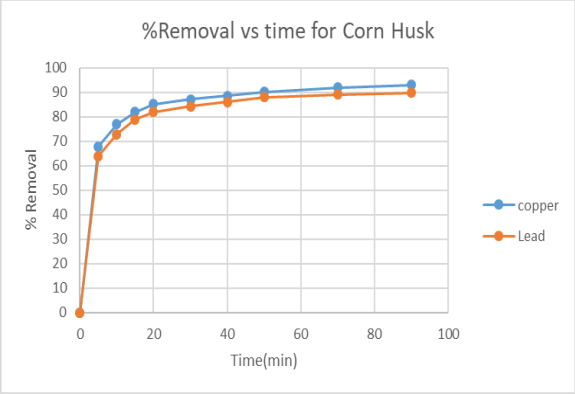

Fig 3.2: Graph of %Removal vs Time for corn husk

From the above graph we can say that adsorbent made from corn husk removes copper more effectively compared to lead.

3.3 Experimental Data for Isotherm of Corn Husk

3.3.1 Experimental data for copper

Table 3.5: Isotherm data for copper using corn husk

Initial Conc.(C) (mg/lit)	Q = X/M	% Removal	C/Q
20	17.322	86.61	1154.601
40	32.368	80.92	1235.788
60	45.138	75.23	1329.257
80	46.632	58.29	1715.56
100	50.31	50.31	1987.676
120	53.388	44.49	2247.696
140	57.918	41.37	2417.211
160	60.528	37.83	2643.405
180	61.038	33.91	2948.983
200	61.86	30.93	3233.107

3.3.2 Experimental data for lead

Table 3.6: Isotherm data for lead using corn husk

Initial Conc.(C) (mg/lit)	Q = X/M	% Removal	C/Q
20	17.782	88.91	1124.733
40	34.036	85.09	1175.226
60	48.546	80.91	1235.941
80	48.728	60.91	1641.767
100	51.32	51.32	1948.558
120	54.192	45.16	2214.349
140	59.038	42.17	2371.354
160	62.848	39.28	2545.825
180	63.63	35.35	2828.854
200	64.38	32.19	3106.555

3.3.3 Isotherm of corn husk

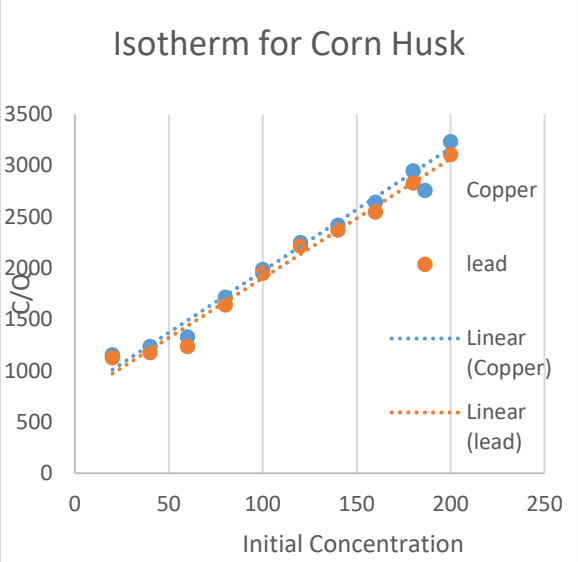

Fig 3.3: Langmuir isotherm of corn husk

From the above graph it shows that experimental data are fitted into following Langmuir isotherm equation

$$C/Q = 12.01 \times C + 770.17$$

Where,

C = Initial concentration of the solution

Q = X/M = grams of adsorbate per gram of adsorbent

3.4 Experimental Data for Isotherm of Rice Husk

3.4.1 Experimental data for copper

Table 3.7: Isotherm data for copper using Rice husk

Initial Conc.(C) (mg/lit)	Q = X/M	% Removal	C/Q
20	16.262	81.31	1229.861
40	31.156	77.89	1283.862
60	43.548	72.58	1377.79
80	44.896	56.12	1781.896
100	49.73	49.73	2010.859
120	51.852	43.21	2314.279
140	55.482	39.63	2523.341
160	57.792	36.12	2768.549
180	59.364	32.98	3032.141
200	62.48	31.24	3201.024

3.4.2 Experimental data for lead

Table 3.8: Isotherm data for lead using rice husk

Initial Conc.(C) (mg/lit)	Q = X/M	% Removal	C/Q
20	19.146	95.73	1044.605
40	37.208	93.02	1075.038
60	55.104	91.84	1088.85
80	56.328	70.41	1420.253
100	58.32	58.32	1714.678
120	58.932	49.11	2036.245
140	60.508	43.22	2313.744
160	64.784	40.49	2469.746
180	67.014	37.23	2686.006
200	67.78	33.89	2950.723

3.4.3 Isotherm of rice husk

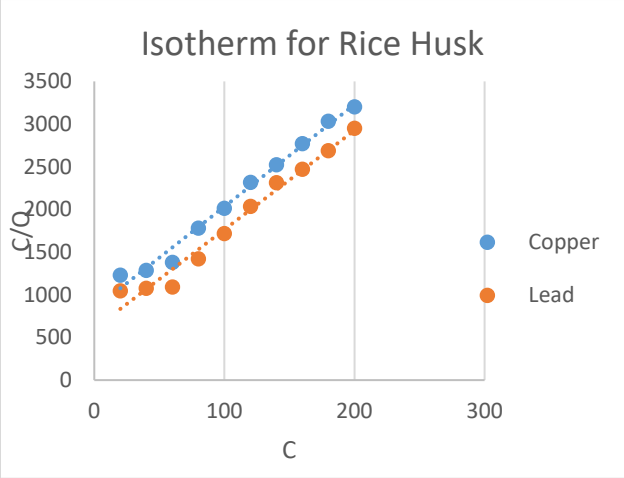

Fig 3.4: Langmuir isotherm of rice husk

From the above graph it shows that experimental data's are fitted into following Langmuir isotherm equation

$$C/Q = 11.958 \times C + 837.03$$

where,

C = Initial concentration of the solution

Q = X/M = grams of adsorbate per gram of adsorbent

CONCLUSION:

By doing this experiment we have concluded that with increase in contact time conc. of lead and copper decreases in water in presence of Ash of Corn Husk and Rice Husk as Adsorbent and the experimental data that has been obtained also says the same. Also from the obtained experimental data it is being concluded that when the mixed adsorbent of Corn and Rice husk is made both ions i.e. lead and Copper are effectively removed and Rice husk removes lead effectively and Corn husk removes copper effectively. Hence mixed Ash from Corn Husk and Rice Husk can be used as effective adsorbent for removal of lead and copper ions simultaneously from water.

ACKNOWLEDGMENT: The authors wish to thank head of chemical engineering department for valuable and helpful discussions. We greatly acknowledge technical and economical support from the GCET college.

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PRAJÑĀ - **Journal of Pure and Applied Sciences Vol. 27-28 (2019-20)**
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