THE OPTIMIZATION AND QUANTIFICATION OF CHITINASE ENZYME PRODUCTION FROM THE SOIL BACTERIA OF TITHAL COASTLINE, VALSAD, GUJARAT

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Abstract
Chitin is the most prevalent renewable polymer in the ocean and a crucial source of carbon and nitrogen for marine species. The goal of this study was to characterise chitinase-producing bacteria isolated from the soil of Tithal coastline in Valsad, Gujarat, and to investigate conditions affecting chitinase production by various factors such as inoculum size, incubation temperature, medium pH, substrate concentration, carbon source (glucose, sucrose, lactose, mannitol, and Fructose), nitrogen source (yeast extract, peptone, urea The quantification method was Lowry's test, and the standard protein was Bovine Serum Albumin (BSA). Several process factors are also used to optimise the chitinase production medium. The release of reducing sugar from colloidal chitin was used to perform the chitinase assay.

Keywords: chitinase, production, soil bacteria, Tithal

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1. INTRODUCTION

Chitin is plentiful and widely distributed in nature. It is, after cellulose, one among the most abundant biopolymers on Earth. The shells, exoskeletons, and stomach linings of arthropods all include chitin (crustaceans and insects). It's also found in the cell walls of several fungi, including yeasts, and in the structural frameworks of some Protista and worm eggs. Although many microbial genomes contain genes encoding chitinolytic enzymes, which have been extensively studied, research on microbes that utilize insoluble chitin as a carbon source in biotechnology is limited [1]. According to earlier studies, disease losses in the field account for around 35 percent of crop yields, whereas postharvest losses account for closer to 15 percent of total yields [2]. Insects, weeds, and diseases are the primary causes of crop loss. As a result, crops all over the world are fully reliant on the application of fungicides and pesticides to prevent loss, but the main issue with doing so is that the target organisms often develop resistance to these chemicals. Management measures can help to lessen this effect, but they won't stop resistance from developing altogether [3]. Pesticides' impact on non-target creatures as well as human health has been examined extensively, with alarming findings. Fungicide leaching into ground water harms aquatic ecosystems and drinking water supplies [4], and pesticide use has been connected to a variety of human health issues [5]. Pesticides were discovered in honey samples recently [6]. The development of alternate ways to control both pre- and postharvest illnesses has been motivated by public concern about chemical residues as well as pathogen development of fungicide resistance [3].

Chitinases are hydrolytic enzymes that are produced by microorganisms. Firmicutes [7] and certain Proteobacteria [8] are well known for producing and using chitinolytic enzymes. Antibacterial, antifungal, insecticidal, and nematicidal activity is exhibited by microbial *chitinases*, which weaken and breakdown the cell walls of many pests and diseases [9]. Chitinolytic enzymes will become a more visible and essential tool for addressing the environmental and human risks posed by synthetic pesticides and fungicides. As a result,

chitinolytic bacteria have the potential to replace the more hazardous practice of using insect- and fungal-killing pesticides.

The goal of our study is to better understand the role of *chitinases* in bacterial inoculants effects. We review the research on chitin-degrading enzymes in microorganisms with biocontrol potential and outline the various functions and modes of action that a microbial inoculants should have against diseases, both in the field and during postharvest storage. We also present numerous assays for screening hitherto unstudied microbial species for chitinolytic activity, which will aid in their characterization and usage as pesticide alternatives.

Chitinolytic enzymes are synthesized by most common bacterial and fungal microbiota, and some undiscovered species effectively dissolve this chitin polymer. According to researchers, marine settings are the primary source of *chitinase*-producing microorganisms, which are predominantly bacterial species [10], with just 4% of strains classified [11]. *Bacillus, Aeromonas, Serratia, Enterobacter, Erwinia, Chromobacterium, Flavobacterium, Arthrobacter,* and *Vibrio* are some of the genera that have been identified as *chitinase* producers in the marine ecosystem [12]. *Streptococcus, Clostridium, and Eubacterium genera* isolated from whale wastes [13]; *Bacillus licheniformis* isolated from food industry liquid waste [14]; *Streptomyces* and *Serratia* isolated from crustacean residues [15]; and *Bacillus amyloliquefaciens* and *Acinetobacter johnsonii* isolated from shrimp residues. Because they are aerobic, *chitinase* manufacturers are rarely isolated from aquatic ecosystems. Many academics are focusing on identifying *chitinase* manufacturers in order to pave the way for cost-effective and environmentally favorable breakdown of shrimp leftovers. As a result, the current research focused on isolating and identifying a *chitinase*-producing strain utilizing 16S rRNA sequencing. Hyper*chitinase* was discovered in a mutant strain.

Streptomyces, Bacillus, and *Pseudomonas* species are among the most common bacteria utilised as biocontrol agents [17]. Given the large number of chitinolytic bacteria under investigation, as well as the fact that current pathogen control tactics are detrimental, developing new pesticides based on biocontrol bacteria could be a viable option. Pesticides have well-documented and well-documented negative environmental consequences. These chemicals not only influence organisms outside of the target organisms in the environment, but they can also migrate within and outside of the application site [18]. Furthermore, when pesticides were introduced to the soil in large doses, [19] found a decrease in microbial activity. It was not always possible to recover microbial activity.

Insect growth has also been shown to be influenced by *chitinases;* when larvae come into touch with *chitinases,* their feeding rate and body weight both fall, resulting in death. The weakening of the peritrophic membrane, which borders the gut epithelium of larvae and is made up primarily of chitin [20], is thought to be the cause of these symptoms. The peritrophic membrane of *Orgyia pseudotsugata* was found to be destroyed by *chitinases* by Brandt et al. [21], and this effect was later seen in vivo with *Spodoptera littoralis* and *E. coli* that expressed the *Serratia marescens endochitinase ChiAII* [22]. Insects and pathogenic fungus have developed resistance to both chemical pesticides and B. thuringiensis toxin in recent studies. Bacteria of several orders have been discovered to be excellent biocontrol agents in this regard [23]. They are one of the most important taxa in the chitinolytic community of soil microbes [24,25]. Bai et al. [26] discovered that the *Actinobacteria* were responsible for about half of the terrestrial *chitinase*-containing bacterial genomes they examined from public databases.

The ability of *Streptomyces* species to penetrate solid chitin pieces with their hyphae allows them to degrade solid chitin fragments quickly [27,28]. Various chitinous compounds, such as chitosan and shrimp waste, were discovered to be used as nutritional sources by a *Streptomyces rimosus* strain isolated from agricultural soil in central Poland [29]. Kawase

et al. [30] identified 13 different *chitinases in Streptomyces coelicolor*, including 11 GH18 kinds (A, B, and C) and two GH19 *chitinases*. A variety of chitinases have been discovered in other *actinobacteria. Nocardiopsis prasina*, for example, secreted three *chitinases* in the presence of chitin: ChiA, ChiB, and ChiB [31]. The ChiB protein's catalytic domain was discovered to be comparable to *Streptomyces* GH19 *chitinases*, and it demonstrated significant antifungal activity, as expected of GH19 type *chitinases.*

Streptomyces is one of the most well-studied genera in terms of *chitinase* activity, however many other *actinobacteria* have similar abilities but are less well-studied. *Streptomyces rimosus* purified chitinase inhibited *Fusarium solani* and *Alternaria alternata* in vitro. *Streptomyces viridificans* destroyed the fungal cell walls of *Rhizoctonia, Colletotrichum, Aspergillus, Fusarium, Sclerotinia, Curvularia,* and *Pythium* in vitro, as well. *S. hygroscopicus* was shown to be hostile to *Colletotrichum gloeosporioides* and *Sclerotium rolfsii* among the *Streptomyces* species isolated from *rhizosphere* soils by [32]. The most potent fungal inhibitors were culture filtrates acquired during a growth phase when *chitinase* and 1,3-glucanase production was maximum.

When they generated *chitinase*, actinomycete isolates from Jordanian soil samples showed in vitro fungicidal action against *Sclerotinia sclerotiorum mycelial* growth and *sclerotia* development. Isolates that didn't make *chitinase*, on the other hand, only had a fungistatic effect [33]. *Streptomyces roseolus GH18 chitinase* demonstrated a significant inhibitory effect on fungal hyphal expansions.

The mycelial growth of *Rhizoctonia solani*, which causes sugar beet damping-off disease, was inhibited in vitro by two *chitinolytic streptomycetes*. In both infected and non-infected situations, soil treatment with either isolate totally stopped the disease and considerably increased seedling growth. All bacteria-containing treatments enhanced shoot and root dry biomass when compared to the controls [34]. Gherbawy and colleagues [35] discovered seven strains that produced GH19 *chitinases* that were antifungal against *Fusarium oxysporum, Pythium aristosporum, Colletotrichum gossypii,* and *Rhizoctonia solani.*

Streptomyces viridodiasticus also produced antifungal compounds that inhibited pathogen growth in vitro. All three actinobacterial isolates generated substantial hyphal plasmolysis and cell wall lysis when injected with *S. viridodiasticus* isolates in living *Sclerotinia* minor mycelial mats cultured in a carbon-free salt solution. In addition, under controlled greenhouse circumstances, the isolates, alone or in combination, considerably reduced illness incidence [36]. *Streptomyces cavourensis* has been identified as a possible biocontrol agent for anthracnose in pepper, owing to a combination of *chitinolytic* enzymes and the antifungal chemical 2-furancarboxaldehyde [37]. *Bacillus thuringiensis* is a well-known biocontrol agent that has been used to control pests in agriculture and disease-carrying insect vectors for decades.

There have been numerous *B. thuringiensis* strains identified that express *chitinase* constitutively [38]. *Bacillus thuringiensis* isolates were obtained from tomato roots by *Hollensteiner et al*. [39], and the isolates showed antifungal activity against *Verticillium spp.* in vitro. Only isolates carrying one or two putative chitinase genes were unable to develop. Genes encoding the antifungal *siderophore bacillibactin* were found in all of the isolates, and one isolate had a gene encoding the antibiotic *zwittermicin A*, indicating that these *chitinolytic* bacteria had diverse antifungal mechanisms [40,41]. If you're looking for a unique way to express your creativity *Brevibacillus* laterosporus had two chitinolytic enzymes with GH18domains but different C-terminal domains, according to the research. To ensure effective hydrolysis, the latter determines substrate specificity.

Some investigations also reveal a synergistic interaction between Bacillus *thuringiensis endotoxins and chitinases* [42], suggesting that mixed formulae containing bacterial consortia for inoculation could be more efficient for

biocontrol than single strains. *B. thuringiensis* produces two *chitinases* that improved the insecticidal efficacy of Bt crystal protein against *Spodoptera exigua and Helicoverpa armigera* larvae and nearly entirely suppressed the germination of R. solani and B. cinerea spores [43]. Bacillus cereus was isolated from the eggplant rhizosphere [44]. These researchers discovered that bacterial suspensions, supernatants, and a diluted *chitinase* solution all inhibited fungal spore germination. The supernatant and purified enzymes were less effective than a suspension of the strain's cells in lowering the severity of *Verticillium* wilt on eggplant in greenhouse studies; the cell suspension decreased symptoms by 70% in 14 days.

Bacillus pumilus, another firmicute, was also discovered to have high chitinolytic activity. It reduced the growth of *Scirpophaga incertulas*, a rice pest [45], as well as other taxa of pathogenic fungi of agronomic importance.

When tested on colloidal chitin, *Paenibacillus illinoisensis*, a bacteria isolated from Korean coastal soil, showed high in vitro chitinolytic activity. The root-knot nematode (Meloidogyne incognita) had its eggshell distorted and killed as well [46]. Singh et al. [47] discovered that *Helicoverpa armigera* may be controlled using *Paenibacillus sp. D1*, a high-producing chitinase bacteria. This strain had a 40% mortality rate in the larvae, and when paired with acephate (a pesticide), it had a synergistic effect. Numerous more proteobacteria, in addition to the microbes described previously, are positive biocontrol agents that have been thoroughly researched.

Serratia marescens' chitinolytic mechanism has received a lot of research [28]. ChiA, ChiB, ChiC1, and ChiC2 are among the GH18 *chitinases* produced by *S. marescens*, with ChiC2 arising from a post-translational alteration of ChiC1 [48,49]. These enzymes work in distinct ways, allowing for faster chitin hydrolysis [50].

Botrytis cinerea conidiospores were suppressed and germ tube development was deformed in vitro by *Serratia marescens endochitinase* and chitobiase. When combined with *chitinases*, prodigiosin, a red pigment generated by *S. marescens*, improved inhibition [51]. *Phytophthora capsici* causes cucumber damping-off disease, which is controlled by this pigment. Disease symptoms were unaffected by mutants that were deficient in prodigiosin production [52].

2. MATERIALS AND METHODS

The chitin colloidal solution was prepared, and the method is discussed in this section. This is followed by the optimization of the production of *chitinase* . The assay method of chitinase is also discussed in this part.

A. Colloidal chitin preparation: The modified Hsu and Lockwood method was used to produce colloidal chitin from the chitin (Hi Media). In a nutshell, 40 g of chitin powder was slowly mixed with 600 ml of strong HCl and held at 30ºC for 60 minutes while vigorously stirring. Chitin was precipitated as a colloidal suspension in 2 liters of water at 4–10 degrees Celsius. The suspension was recovered using suction filtration on coarse filter paper and then washed in about 5 liters of DW. The process was repeated three times until the suspension's pH reached 3.5. The loose colloidal chitin was utilized as a substrate following the process described above [53, 54].

B. Chitinase production and its optimization: Submerged fermentation was used to improve several process parameters that influence the synthesis of chitinase for the isolate. The optimization was done in a one-factor-at-a-time fashion, with one optimized parameter being followed while additional parameters were optimized. The effort entailed optimizing chitinase production at a few different levels, including inoculums size, inoculums size, inoculums size, inoculums size, inoculums size, and inoculums (1 percent, 2 percent, 3 percent, 4 percent, and 5 percent). Temperatures of incubation (25°C, 30°C, 35°C, 40°C, and 45°C) (4, 6, 8, 10, and 12) Carbon source (Glucose, Sucrose, Lactose, Mannitol, and Fructose) Nitrogen source (Yeast extract, Peptone, Urea, Ammonium sulphate, and Gelatin) Metal ions (Co^{2+}, Ca^{2+}, Cu^{2+}, Mg^{2+}, and Mn^{2+}) Inorganic salts (g/L): 0.7 g KH_2PO_4;

0.3 g K_2HPO_4; 4 g NaCl; 0.5 g $MgSO_4 \cdot 7H_2O$; 1 mg $FeSO_4 \cdot 7H_2O$; 0.1 mg $ZnSO_4 \cdot 7H_2O$; 0.1 mg $MnSO_4 \cdot 7H_2O$ were used to optimize various process parameters in *chitinase* production medium.

The same culture media composition was employed for optimizing inoculums size, incubation temperature, and medium pH. The media was supplemented with various concentrations of colloidal chitin during the optimization of substrate concentration, and 0.2 percent colloidal chitin was added for other parameters before to that. The media was added with 1 percent of each source separately for nitrogen and carbon source optimization. Except for copper, which was given with sulphate, the medium was supplemented with chloride salts of the selected metal at 0.1 mg/L. First, a single colony of the bacterial isolate was inoculated into 25 mL nutritional broth medium and cultured at 30°C until optical density at 600 nm reached 1. Except in the event of inoculums optimization, this pre-culture was utilized to inoculate fermentation medium production medium at a rate of 2% (v/v). After that, the cultures were incubated at 30°C and 150 revolutions per minute. All optimizations were subjected to a 72-hour incubation period. The crude enzyme was prepared from each setup by centrifuging the culture media at 8000 rpm for 5 minutes and using the supernatant in the experiment**.**

C. Assay method of chitinase enzyme: The release of reducing sugar from colloidal chitin was measured using the Chitinase assay, which was slightly modified from [55]. In phosphate buffer, 1 percent (w/v) colloidal chitin was added with equivalent volume of crude enzyme (0.5 mL) (0.1 M, pH 7). The reaction mixture was added to 1 mL of DNS reagent and heated for 5 minutes after being incubated at 37°C for 1 hour. The supernatant optical density was measured at 540 nm after the reaction mixture was spun at 9,000 rpm for 5 minutes. The quantity of *chitinase* that releases one mol of N-acetyl-D-glucosamine (GlcNac) per hour from 1 percent (w/v) colloidal chitin under specific circumstances was defined as one unit (U) of *chitinase*. With the use of an equation established on the standard curve of N-acetyl-D-glucosamine, the optical density value obtained for each assay setup was utilized to calculate the amount of reducing sugar released (GlcNac). The concentration of the reducing sugar released was calculated using the value obtained for the amount released, and the unit of enzyme was estimated using the value obtained for reducing sugar released.

3. RESULTS AND DISCUSSION

First of all, the standard curve of N-acetyl-glucosamine was developed with the concentration range from 0.1 µg/ml to 0.5 µg/ml. The equation obtained from curve was used to estimate the moles of reducing sugar liberated for each setup during the enzyme activity. The results are shown in **Figure 1-15 and in Table 1-8**. Different studies are carried out such as the effect of temperature, pH, substrate concentration, inoculum size and the details are given in the table and figure section.

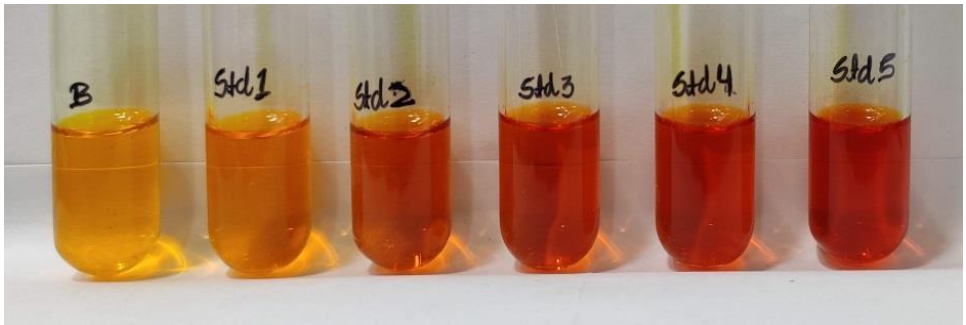

Figure 1. Showing the setup of DNS assay performed for the standard curve formation with N-acetyl-glucosamine

Figure 2. Showing the fermentation media setup after incubation period for the optimization of inoculum size (I)

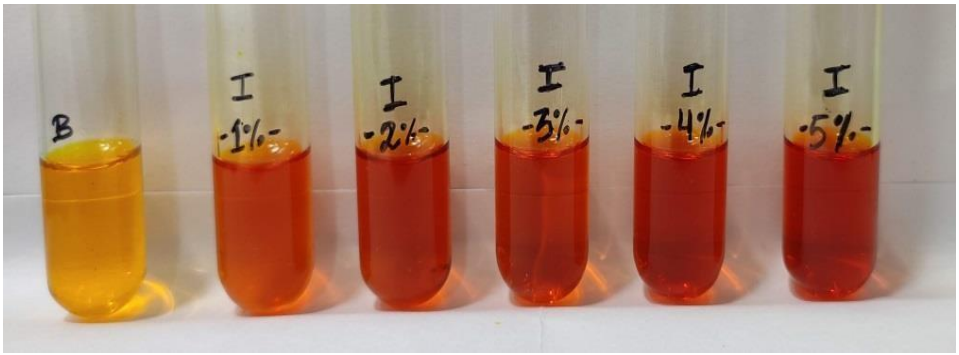

Figure 3. Showing the enzyme activity setup for the optimization of inoculum size (I)

Figure 4. Showing the fermentation media setup after incubation period for the optimization of Temperature (T)

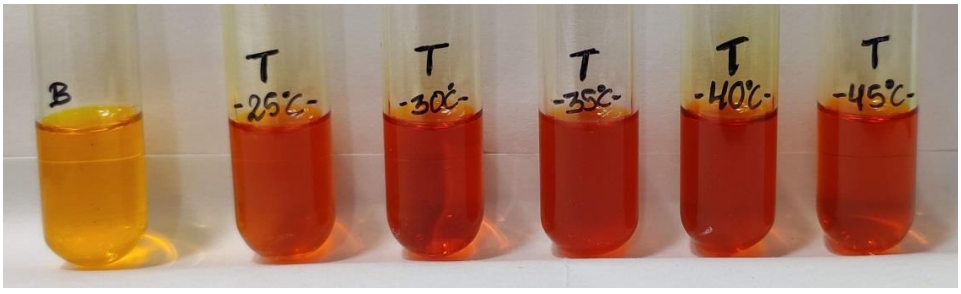

Figure 5. Showing the enzyme activity setup for the optimization of Temperature (T)

Figure 6. Showing the fermentation media setup after incubation period for the optimization of Substrate concentration (C)

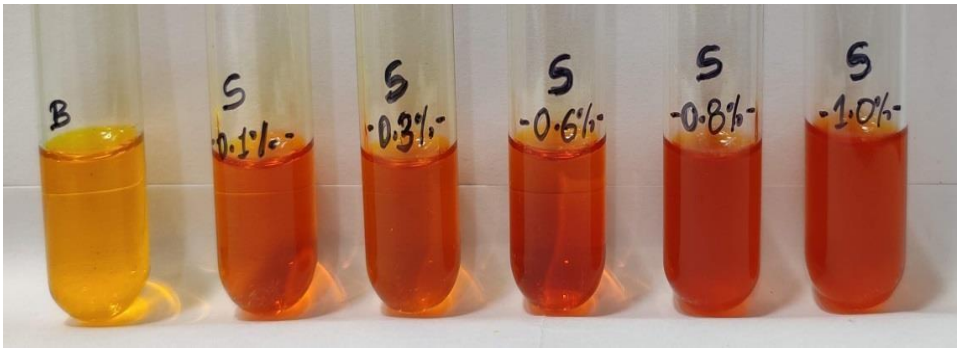

Figure 7. Showing the enzyme activity setup for the optimization of Substrate concentration (C)

Figure 8. Showing the fermentation media setup after incubation period for the optimization of pH (pH)

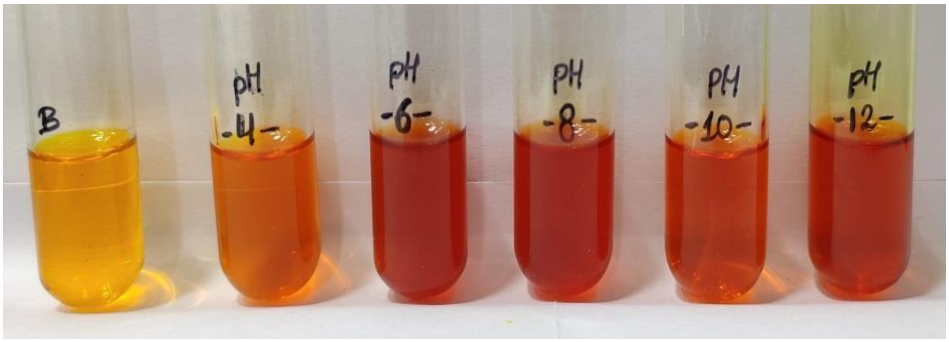

Figure 9. Showing the enzyme activity setup for the optimization of pH (pH)

Figure 10. Showing the fermentation media setup after incubation period for the optimization of Carbon source (C)

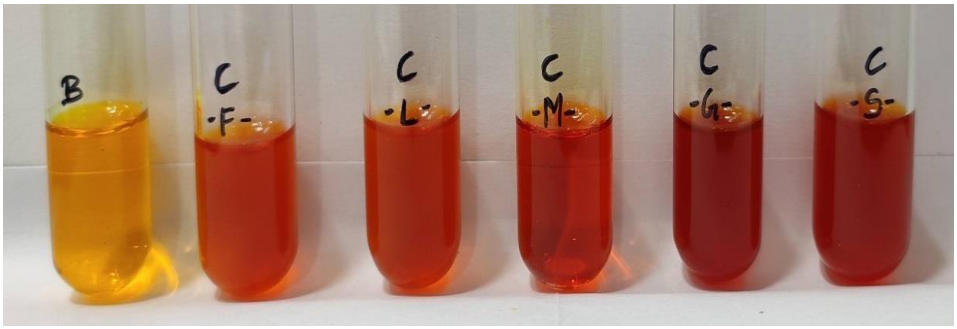

Figure 11. Showing the enzyme activity setup for the optimization of Carbon source (C)

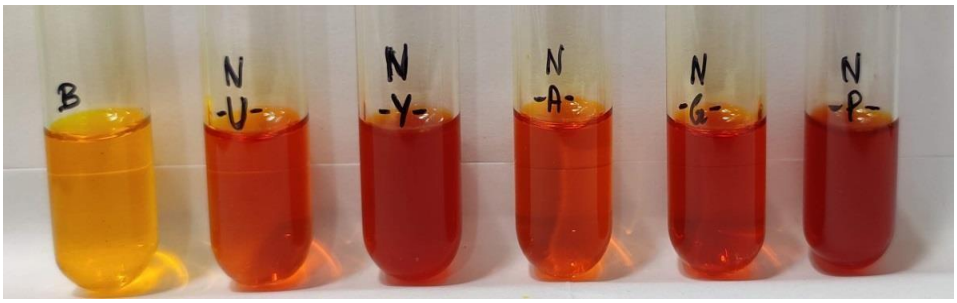

Figure 12. Showing the fermentation media setup after incubation period for the optimization of Nitrogen source (N)

Figure 13. Showing the enzyme activity setup for the optimization of Nitrogen source (N)

Figure 14. Showing the fermentation media setup after incubation period for the optimization of Metal ions (M)

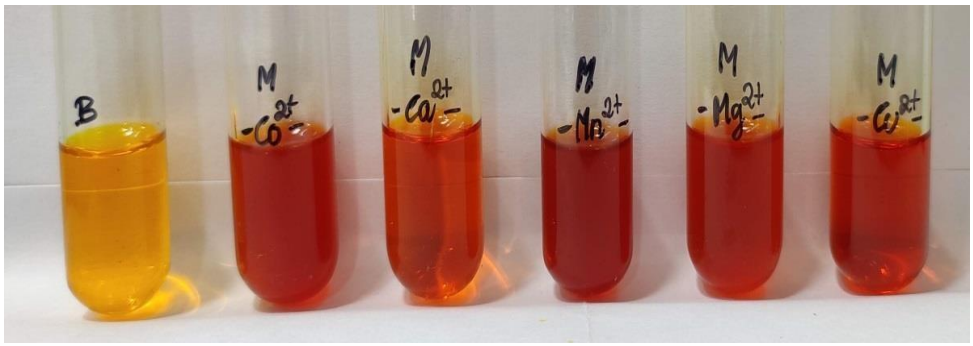

Figure 15. Showing the enzyme activity setup for the optimization of Metal ions (M)

Table 1. Showing the value for the Optical Density O.D. at 540 nm obtained for different concentration of N-acetyl-glucosamine

Name	Concentration (µg/ml)	O.D. at 540 nm
Blank	0	0
Std1	0.1	0.082
Std2	0.2	0.171
Std3	0.3	0.252
Std4	0.4	0.335
Std5	0.5	0.419

Table 2. Showing the value for the OD at 540 nm and chitinase concentration obtained for optimization of inoculum size

Parameter	O.D. at 540 nm	Chitinase (U/ml)
1%	0.201	1.08
2%	0.219	1.17
3%	0.239	1.28
4%	0.246	1.32
5%	**0.268**	**1.44**

Table 3. Showing the value for the OD at 540 nm and chitinase concentration obtained for optimization of incubation temperature

Parameter	O.D. at 540 nm	Chitinase (U/ml)
25ºC	0.254	1.36
30ºC	0.262	1.41
35ºC	**0.270**	**1.45**
40ºC	0.251	1.35
45ºC	0.248	1.33

Table 4. Showing the value for the OD at 540 nm and chitinase concentration obtained for optimization of substrate concentration

Parameter	O.D. at 540 nm	Chitinase (U/ml)
0.1%	0.265	1.42
0.3%	0.270	1.45
0.6%	0.276	1.48
0.8%	**0.284**	**1.52**
1.0%	0.280	1.50

Table 5. Showing the value for the OD at 540 nm and chitinase concentration obtained for optimization of medium pH

Parameter	O.D. at 540 nm	Chitinase (U/ml)
4	0.255	1.37
6	0.282	1.51
8	**0.288**	**1.55**
10	0.262	1.41
12	0.257	1.38

Table 6. Showing the value for the OD at 540 nm and chitinase concentration obtained for optimization of carbon source

Parameter	O.D. at 540 nm	Chitinase (U/ml)
Glucose	**0.395**	**2.12**
Sucrose	0.388	2.09
Lactose	0.357	1.92
Mannitol	0.315	1.69
Fructose	0.336	1.81

Table 7. Showing the value for the OD at 540 nm and chitinase concentration obtained for optimization of Nitrogen source

Parameter	O.D. at 540 nm	Chitinase (U/ml)
Yeast extract	0.488	2.62
Peptone	**0.499**	**2.68**
Urea	0.419	2.25
Ammonium sulfate	0.399	2.14
Gelatin	0.428	2.30

Table 8. Showing the value for the OD at 540 nm and chitinase concentration obtained for optimization of metal ions

Parameter	O.D. at 540 nm	Chitinase (U/ml)
Co^{2+}	**0.632**	**3.40**
Mg^{2+}	0.592	3.19
Mn^{2+}	0.576	3.10
Cu^{2+}	0.493	2.65
Ca^{2+}	0.489	2.63

We got outstanding results from our study in terms of inoculum size, temperature study, and the optimum temperature was found to be 35°C, substrate concentration was varied, and its influence was verified, and the optimum substrate concentration was found to be 0.8 percent. The influence of pH on chitinase production was also investigated, with the greatest results achieved when the pH was alkaline, i.e. 8. A study was also conducted to determine the optimal carbon source, which was discovered to be glucose. Peptone was identified as the nitrogen source. Cobalt (Co^{2+}) was discovered as the metal. For the most efficient generation of chitinase, the inorganic salt medium supplemented with glucose, peptone, and cobalt chloride is best. Below is a table of the outcomes (table 9).

Table 9. The Final Optimized conditions to produce Chitinase enzyme by the given isolate

The Final Optimized conditions to produce Chitinase enzyme by the given isolate are:		
1	Inoculums Size	5%
2	Temperature	35ºC
3	Substrate concentration	0.8%
4	pH	8
5	Carbon source	Glucose
6	Nitrogen source	Peptone
7	Metal ions	Cobalt (Co^{2+})

4. CONCLUSIONS

We have obtained excellent and fascinating results from our investigation on the optimization and measurement of *chitinase* enzyme synthesis from soil bacteria along the Tithal coast of Valsad, Gujarat. For the manufacture of *chitinase* enzyme, the optimal temperature, pH, substrate concentration, carbon supply, nitrogen source, and metal ions were examined. For the most efficient generation of *chitinase*, the inorganic salt medium supplemented with glucose, peptone, and cobalt chloride is best. The discovery of chitinase synthesis from soil bacteria has opened new possibilities.

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