Research Article CODEN: IJPRNK Impact Factor: 5.567 ISSN: 2277-8713
Gayatri Patel, IJPRBS, 2017; Volume 6(3): 1-17 IJPRBS

INTERNATIONAL JOURNAL OF
PHARMACEUTICAL RESEARCH AND BIO-SCIENCE

ISOLATION, IDENTIFICATION AND OPTIMIZATION OF PROTEASE PRODUCING *STENOTROPHOMONAS SPP*. FROM DAIRY REGION SOIL

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Accepted Date: 09/04/2017; Published Date: 27/06/2017

Abstract: The objective of present study was to screen and isolate protease producing bacteria from dairy region soil from mehsana. Sample were serially diluted and o.5 ml of sample was spread on skim milk agar plate at 37 ℃ for 24 hours .Total 5 colony from dairy region soil showed clear zone around the colony indicating protease activity. Morphology characterization by gram staining and organisms was identify by biochemical test and by 16S-rRNA identify strain is *Stenotrophomonas spp*. Moreover the physiological character were like pH , temperature ,carbon source ,nitrogen sources were study for optimization of enzyme. The protease shows maximum pH is 9.5, The maximum temperature is 37ºC. The maximum carbon source glucose. the nitrogen source is yeast extract. The unknown concentration of crude enzyme was using casein-folin method. These bacterial isolate can be used as biotechnological tool for industrial purpose.

Keywords: Soil sample, Protease enzyme, Casein-folin method *Stenotrophomonas spp.*

PAPER-QR CODE

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Access Online On:

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How to Cite This Article:

Gayatri Patel, IJPRBS, 2017; Volume 6(3): 1-17

INTRODUCTION

Protease is also known as peptidase or proteinase is any enzyme that performs proteolysis that is start to protein catabolism by hydrolysis of the peptide bonds that link amino acids together in a polypeptide chain. Protease is mostly live in plants, animals, virus, and bacteria. To creating eco-friendly environmental products and product lay out chemicals processes is starts interchange by enzyme such as protease *(Nigam e.t al 2012).*protease sources is gain from plants and animals, microbes sources *(mukesh kuma e.t al 2012*). Microbial proteases has the best sources because of to their rapid growth the fix spaced need for cell cultivation and which the enzyme can be genetically manipulated to creat new enzyme for different use *(Nigam et.al 2012)* microbial protease are the biggest group that has 60% of the world wide in the enzyme market *(Raydaet***.ai** 2012**)** thus on large search of proteases from microbial sources has been an angling process for many few years.

The microbial enzyme that has showing optimal activities at high range of, temperature, pH, and salt concentration that has the good significance *(Nigam et.al 2012).* Protease can be acidic, neutral or alkaline based on their activities at various temperature *(Narsimha et.ai 2011).*

Alkalophilic proteases playing significant role in detergent industries another acidophilic protease playing best role in leather tanning process, food industries and x-ray films.

Some of fungi likes genus *Aspergillus, mucor, rhizopus* and bacteria likes *clostridium, bacillus*, and *pseudomonas* are popular to produce protease. the *bacillus* strain are gain from alkaline protease. the huge Amount alkaline proteases is secret by the *bacillus* strain has important proteolysis activities and stability at desire high pH and also temperature ***(****Kuberan et.al 2010*)

The widely sources of the genus *bacillus* is mostly commercial proteases .mostly nutrient alkaline extracellular proteases for an enzyme to be utilize as detergent additives .the typical detergent ingredient is more stable and active likes surfactants, builders, fillers, fabric softeners, bleaching agent and various other formulation aids.

The optimum condition of *bacillus cercus* producing protease that shows enzymatic maximum activity are at 50c and PH 10.Tha peptide is hydrolysis by the protease and has many various application of food complementary of beasts and poultries ,leathering , oil manufacturing industries, alcohol production industries ,bakery, beer industries .*(Kuberan et.al .2010)*

The Bacillus subtitles group gain from the production of enzyme are involve in the treatment of bums and wounds oral administration of proteases produces an anti-inflammatory response in burns patient and quickly the process of healing .one of the major weakness affecting the stability in wide PH range but it sometime thermo labile .so that it has to be describe to find for

new proteases with original properties form as many various source as possible *(Abdelnasser et.al. 2007*).

MATERIALS AND METHODS

Sample collection

The soil sample was collected from dairy region of Mehsana. Soil were taken from 2-3 cm depth and kept in plastic bag.

Enrichment of Bacteria

For enrichment to take 50 ml nutrient broth in flask and put in shaking incubator for 24-48 hours.

Screening and Isolation of protease producing Bacteria

Protease producing organisms was isolated by spreading technique.0.5 ml of each soil dilution sample are spread on skim milk agar plate. The zone of hydrolysis surrounding the colony which indicates the protease producing bacteria and the strain was selected for further use.

Maintenance of the culture

The pure organisms are transfer on slant and store at 4ºC .The bacteria was culture monthly.

Morphological and Biochemical characterization

To know the morphological characterization by Gram's staining and Biochemical characterization by indole production, methyl red, Vogues Proskauer's, citrate utilization, triple sugar iron, nitrate reduction, catalase, oxidase, gelatin liquefaction, urea hydrolysis etc.

Cultural characterization

The isolates were observed under the microscope to obtain the colony morphology i.e. colour, shape, size, nature of colony and pigmentation (Dipali Parmar2012 & Quang D,2000)

Inoculation preparation

For inoculation to take one loop ful culture of bacteria and inoculate the nutrient broth and put in shaker for 24 hours.

Protease production

Take 5 ml of inoculation medium and inoculate the protease production medium containing the glucose1%, 0.5% casein, 0.55%yeast extract, 0.2%K2HPO4, 1%Na2Co3, 0.2%MgSo4.7H2O pH - 8.put in shaker for 24-48 hours.

Extraction of enzyme from fermentation broth

For the extraction to take 3ml of fermentation broth was taken in centrifuge tube at the end of incubation time and it was centrifuge the 5000 rpm for 20 min then to remove the pellet and take the supernatant as a crud enzyme.

Optimization of enzyme

Optimization of protease producing using different carbon sources

The effect of carbon sources such as starch, glucose, mannitol, and sucrose on protease production by bacterial isolated was investigated. This carbon sources was added In the fermentation broth containing 1%w/v. the carbon source of original production medium was replaced by carbon sources.

Flask were placed in shaker for 24 hours, 48 hours, 72 hours at the end of incubation to checked the enzyme activity.

Optimization of protease production using different nitrogen sources

The effect of nitrogen sources for protease production such as casein, ammonium sulphate, yeast extract, beef extract, and peptone by bacterial isolated was investigated. The nitrogen sources were added in fermentation broth containing 1%w/v flask were put in shaker for 24 hours ,48 hours and 72 hours at 37ºC after incubation checked enzyme activity.

Optimization of protease production at different PH

The effect of different pH likes 6, 8.5, 9.5 and 10 using 1N HCl and NaOH bacterial was inoculated in fermentation broth. Flasks were put in shaker at 37ºC for 24 hours, 48 hours 72 hours after incubation checked the enzyme activity.

Optimization of protease production using different temperature

The effect of different temperature such as 25ºC, 37ºC, 45ºC and the bacterial isolate was inoculated and put the different place for 24 hours, 48 hours, 72 hours. After incubation checked the enzyme activity.

Protease assay by casein-folin method

Requirement

Equipment-Autoclave, spectrophotometer, pH meter, centrifuge, Laminar Air-flow, Reagent-phosphate buffer, casein, Tri-chloroacetic acid, sodium carbonate, folin-ciocaltue

Other Requirement-flasks, pipette, beaker

Procedure

Proteolytic activity was carried out by casein-pholin method. Culture media was centrifuge at 7200 rpm for 10 min and supernatant was used as enzyme sources.

However, 1%casein in 0.1M phosphate buffer and PH-7.0. It was used as substrate .1 ml of each solution enzyme and substrate was incubated 50ºC for 60 min.

The reaction was determined by adding 3 ml of Tri-chloroacetic acid .one unit of protease activity was defined as the increase of 0.1 unit optical density at 1 hour incubation time. Then centrifuge 5000 rpm for 15 min. From this o.5 M of supernatant was taken to this 2.5 ml of 0.5 M sodium carbonate was added mix well and incubated for 20 min.

Then it was added with 0.5 ml of folin phenol reagent and absorbance was read at 660 nm using by spectrophotometer. The amount of protease produced was estimated and expressed in microgram of Tyrosine released under standard assay condition.

Calculation:

Enzyme activity = $\frac{\mu \text{ mol tyrosine equivalent x total volume of assay}}{\text{Volume of enzyme x incubation time x use in colour development}}$ (U/ml)

Protein estimation by folin-lowry method

Requirement-folin reagent, standard protein solution (BSA), Alkaline-copper reagent.

Procedure

First to take clean test tube and pipette the amount of protein solution according to 0.2 ml, o.4ml, 0.6ml, 0.8ml, 1.0ml.

To take 1 ml of enzyme solution

Add require amount of distilled water to make it final volume 1ml.

Then added 5ml of Alkaline copper was added in all tubes.

To put the tubes in incubation at room temperature for 10 min.

Then added o.5 ml folin-phenol solution and incubate for 30 min.

To take the OD at 660nm by using spectrophotometer.

Calculation

Enzyme activity= OD of sample x concentration x O.D of sample

(µg/ml) ml of sample

RESULTS AND DISCUSSION

Total 5 isolates P1 ,P2 ,P3, P4 ,P5 for protease production from soil by using skim milk agar plate.

Figure-1

Figure-2

Figure-3

Observation of staining:-

Microscopic Observation	P1	P2	P3	P4	P5
Shape	Big rods	Cocci	Big rods	Big rods	Big rods
Arrangement	single ,pairs, chain	single ,double and in clusters	single ,pairs, chain	single ,pairs, chain	single, pairs, chain
Gram reaction	+ve	+ve	+ve	+ve	+ve
Spore staining	Spore firming	Spore firming	Spore firming	Non spore forming	Spore firming

Cultural characteristics:-

Characteristics	**P1**	**P2**	**P3**	**P4**	**P5**
Size	Large	Medium	Medium	Large	Large
Shape	Round	Round	Round	Round	Round
Margin	Undulate	Entire	Entire	Entire	Entire
Elevation	Flate	raised	low convex	low convex	Flat
Consistency	Moist	Butyrous	Butyrous	Moist	Butyrous
Opacity	Opaque	Opaque	transparent	Opaque	transparent
Pigmentation	White	crymy white	Orange	crymy white	watery

Biochemical characterization:-

Sugar fermentation

Sugar	P1		P2		P3		P4		P5	
	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
Xylose	+	-	+	-	+	+	+	-	-	-
maltose	+	-	+	+	+	+	+	+	+	+
Glucose	+	-	+	-	+	-	+	-	+	+
manitol	+	-	+	-	-	-	+	+	-	-
Sucrose	+	-	+	+	-	+	+	+	+	+

Test	P1	P2	P3	P4	P5
Methyl red	-	-	+	-	+
v-p	-	-	-	-	-
Indole	+	+	+	+	+
Nitrate production	-	-	-	-	-
Citrate utilization	+	+	-	-	-
Ammonium production	-	-	-	-	-
TSI	-	+	-	-	+

Sugar Fermentation test

Indole production test

Methyl red test

Protease assay

organisms	OD at 660nm	Enzyme activity unit/ml
1	1.548	0.286
2	0.745	0.136
3	0.824	0.151
4	1.232	0.221
5	1.680	0.308

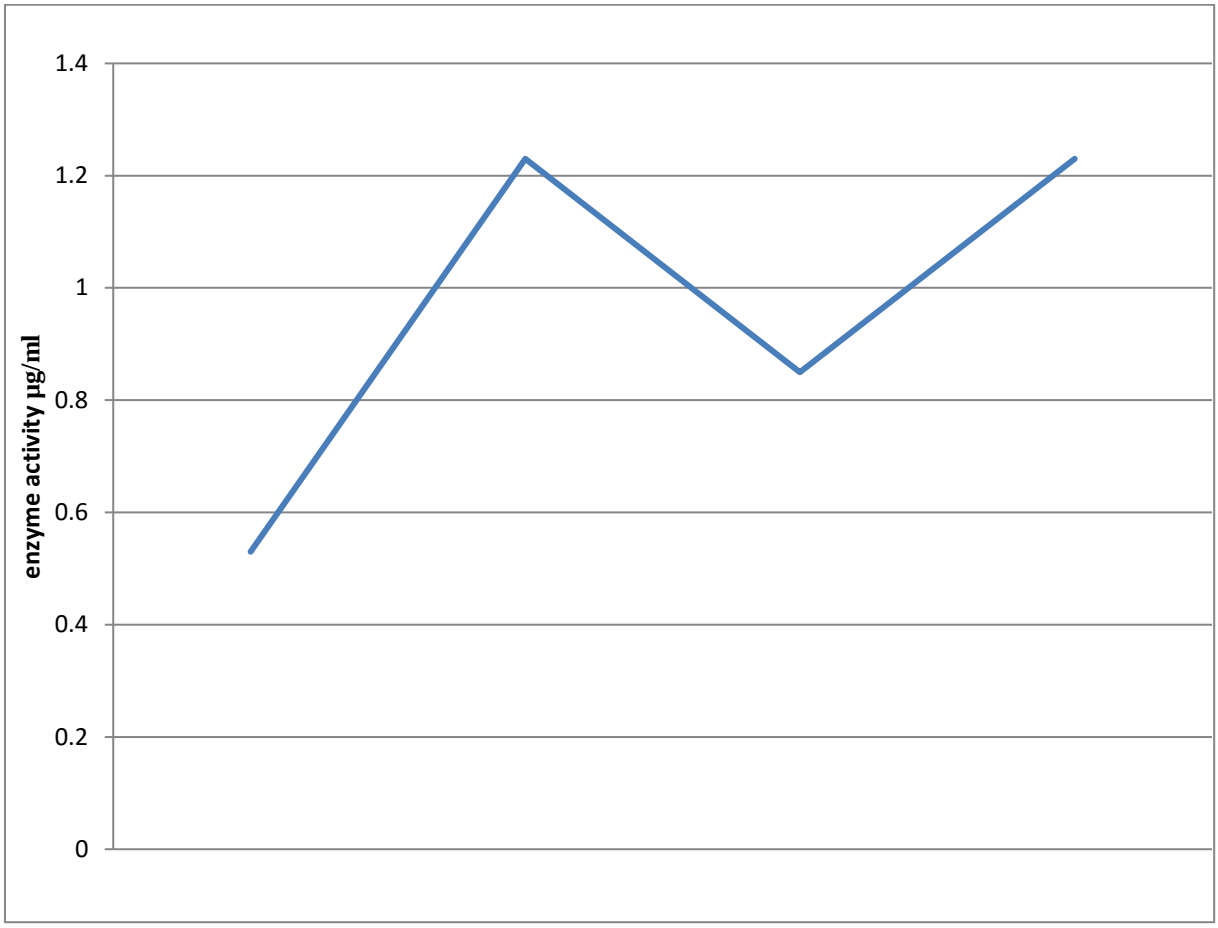
Protein estimation by folin Lawry method

SR NO -1	OD at 660 nm	Enzyme activity(µg/ml)
Standard-1	0.22	-
Standard -2	0.27	-
Standard-3	0.45	-
Standard-4	o.52	-
Standard -5	o.63	-
Enzyme 1	o.51	156.92
Enzyme 2	o.50	153.84
Enzyme 3	o.53	163.07
Enzyme 4	0.58	178.46
Enzyme 5	0.61	187.69

Result-the activity of enzyme is maximum 187.6µg/ml which has the bacteria(p5)

Optimization of enzyme in different carbonsources

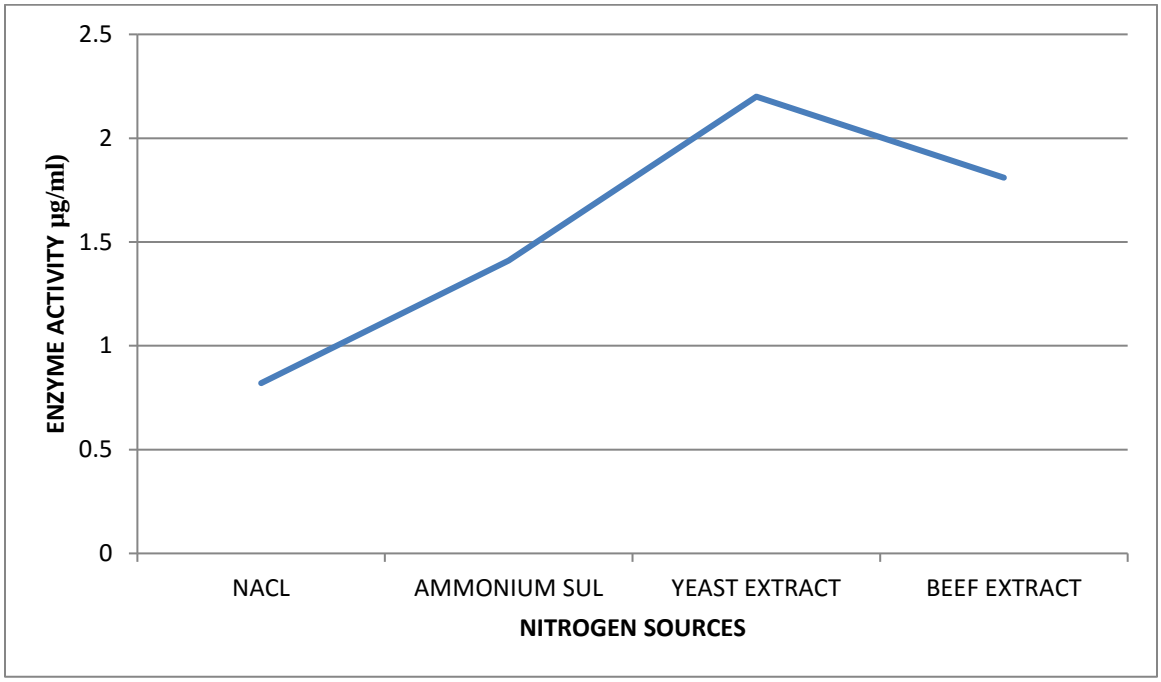
Carbon sources	Enzyme activity
Starch	0.53
Glucose	1.23
Sucrose	0.85
Manitol	1.20



Graph- 1 Enzyme activity--------Carbon sources

4.8 Optimization of different Nitrogen source

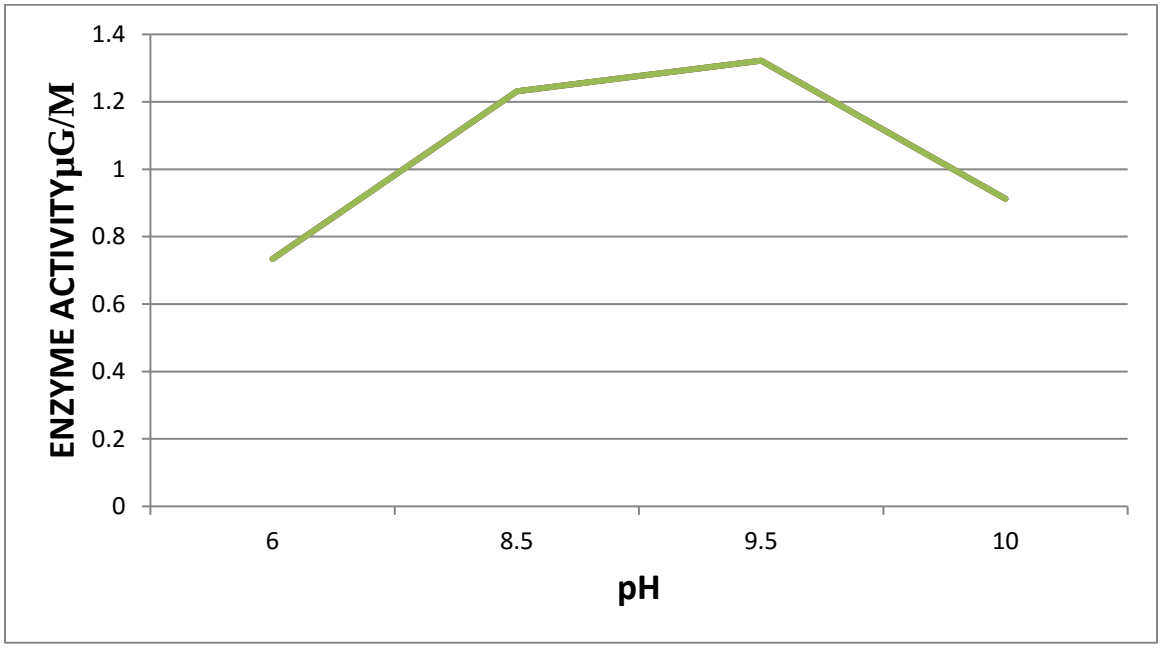
Nitrogen source	Enzyme activity
Nacl	0.82
Ammonium sulphate	1.41
Yeast extract	2.20
Beef extaract	1.81



Graph- 2 Enzyme activity--------Nitrogen sources

4.9 Optimization of enzyme at different PH

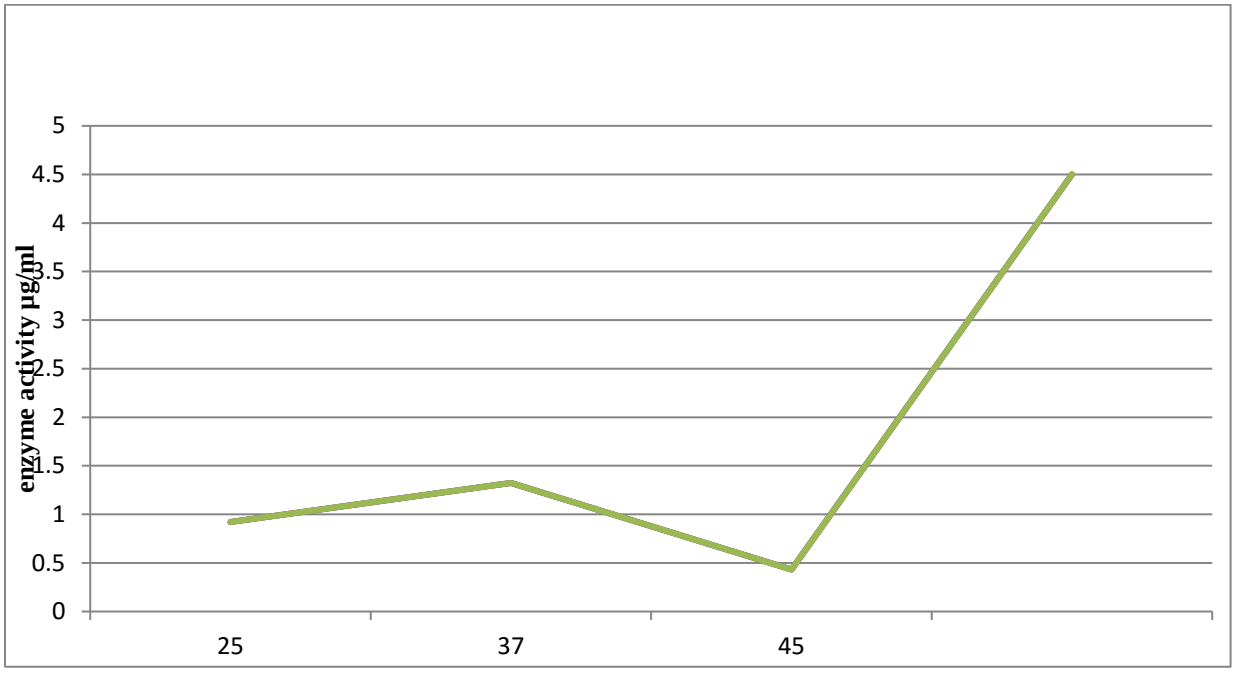
pH	Enzyme activity
6	0.734
8.5	1.231
9.5	1.322
10	0.912



Graph- 3 Enzyme activity--------pH

4.9.1 Optimization of enzyme at different temperature

Temperature	Enzyme activity
25℃	0.921
37℃	1.324
45℃	0.432



Graph- 4 Enzyme activity--------Temperature

CONCLUSION

The main objective of study to proteolysis bacteria that has use in many industrial applications. The proteolytic activity and growth condition of these bacteria are affected by different chemical (types of media used) and physical (pH, temperature) parameters to get proteolysis activity, identification of potential isolate is *Stenotrophomonas spp* and their genbank accession number is KY79273 0optimization of physical and chemical condition for potential protease producing bacteria should be a surgical task.

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