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INVESTIGATING ALPHA AMYLASE ENZYME PRODUCTION BY HUMAN MICROBIOTA

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Key words : *Human microbiota, Amylase enzyme production, 16S rRNA Gene sequencing, Enterobacter sp. and Bacillus* sp.

Abstract – The purpose of the present study was to investigate the amylase enzyme production from human microbiota**.** Human belly button, ear, nasal and armpits were used as source to observe the microbial load. Biochemical test, molecular characterization (16S rRNA Gene sequencing), phylogentics analysis were used to determine the gram positive and gram negative bacteria of normal flora of human. For screening of amylase enzyme producing human microbiota, starch hydrolysis test, enzyme assay, optimum temperature and optimum pH were performed. Isolates of *Enterobacter sp., Bacillus sp.* were found to be prominent human microbiota present in human belly button, ear, nasal and armpits that were biochemically and molecularly characterized. Their 16S rRNA gene sequences were submitted to NCBI Gene Bank and accession number were listed down. Only *Enterobacter hormaechei* (MK503501 & MK503447), *Bacillus mycoides* (MK503615) and *Bacillus subtilis* (MK503496) has shown enzyme production and their optimum pH & temperature were studied. 25 ^{o}C was found to be optimum temperature of .*Enterobacter hormaechei* (MK503501) and *Bacillus mycoides* (MK503615), where as *Enterobacter hormaechei* (MK503447) and *Bacillus subtilis* (MK503496) has shown 50 ^{o}C optimum temperature, respectively. As far as optimum pH wad concerned, specific enzyme activity was found to be present in between pH 5 to 7. Authors would like to suggest using normal flora of human body for the industrial purposes and further to check the pharmaceutical properties like antioxidant and bacteriocin production for future purposes.

INTRODUCTION

Amylase belongs to Endogenous family of enzyme, which has the characteristic property of hydrolyzing within the given oligosaccharide unit. Amylase family is divided into three groups' alpha, beta, and gamma. Activity of Alpha amylase is slower in comparison to beta-amylase. The alpha amylase has characteristic property of hydrolyzing the complex oligosaccharides into simpler smaller units. It has the property of hydrolyzing polysaccharide units into smaller and simpler monosaccharide units by the cleaving of alpha-D [1-4] glucosidic bonds (Ryan, 2011). The enzyme can be isolated from various resources such as plants animals and micro organism but the enzyme isolated from micro organism has a broad spectrum at industrial level due to its stability in the host as compared to that of plants and animals (Gurung *et al.,* 2013). Moreover, Industry Amylase isolated from microorganism is generally preferred over animals and plant due to its stability at extreme temperature and pH, and also it meets the ongoing demands of industries, i.e. it provides the facility of low cost level which can easy be done with the help of microorganism as host (de Souza *et al.,* 2010). Commercial bacterial strains of *Aspergillus sp*. and *Bacillus* sp.,mainly *Bacillus subtilis, B. stearothermophilus*,*B.amyloliquefaciens* and *B. licheniformis* is used for the production of amylase (Subash *et al.,* 2017). *Bacillus* species produces a high amount of extracellular enzymes of which amylases and proteases are being the most top listed in industrial importance Production of thermostable á-

amylase is available from the mesophile *B. licheniformis or Bacillus megaterium* (Morgan *et al.,* 1981 and Jana *et al.,* 1997).

Human microbiota is defined as the aggregate of microorganism that live on human. Large numbers of microorganisms live on and in the various components of the normal skin. After a hot shower, there is an increase in number of microorganism that increases flow of secretion from the skin gland where many reside (Grice *et al.* 2011). Body odor is influenced by the actions of the skin flora, including members of *Corynebacterium* that manufactures enzymes called lipases (Lundström *et al.,* 2010). There have been numerous ways of production of amylase at the industry but in this paper the main focus to be studied how amylase is being produced from human microbiota and that can be utilized for amylase production at industrial level.

MATERIALS AND METHODS

Sample collection

Swabs of Human belly button, ear, nasal and armpits were collected from a volunteer male, age 23 year young. Sterile swab sticks moistened with 1.0 mL of sterile normal saline were rubbed from the Human belly button, ear, nasal and armpits. Collected swabs were inoculated immediaitely in to nutrient broth and were allowed to incubate at 37! for 24hrs (Hulcr *et al.,* 2012).

Isolation and Characterization of Human Microbiota

Serial dilutions were done of collected swabs from Human belly button, ear, nasal and armpits in order to achieve the single colonies. On the basis of size, shape and color, isolates were selected and streaked on nutrient agar to obtain pure culture. Pure cultures were characterized morphologically, and biochemical tests (catalase, coagulase, citrate, urease, TSI and MR-VP) were carried out to authenticate their identity (Sharma *et al.,* 2016).

Molecular Characterization

Genomic DNA was isolated from heavy metal resistant bacteria by lysis buffer and 16S rDNA was amplified by using the universal bacterial 16S rDNA primers, F (5'-AGHGTBTGHTCMTGNCTCAS-3) and R (5'-TRCGGYTMCCTTGTWHCGACTH-3'). PCR products were sequenced by capillary sequencing using ABi 3500 Genetic Analyzer machine as per manufacturer's information. The 16S rRNA gene sequences were compared to identify the most similar sequence alignment. Sequences were matched with previously published bacterial 16S rRNA gene sequences in the NCBI databases using ADVANCED BLAST. Phylogenetic tree was constructed using the Neighbor-Joining method by Phylogeny.fr (http://www.phylogeny.fr/simple_phylogeny.cgi). The sequence was submitted to NCBI – Gen Bank database (Hoffman *et al.,* 1987 & Dereeper *et al.,* 2008).

Screening of amylase enzyme producing bacteria

Isolates were screened for amylolytic activity by starch hydrolysis test on starch agar plates. The microbial isolates were streaked on the starch agar plate and were allowed to incubate at 37 °C for 48hrs. After incubation iodine solution was flooded with dropper for 30 seconds on starch agar plate. Presence of clear zone of hydrolysis indicates positive results. The isolates produced clear zone of hydrolysis were considered as first screening test and they specific alpha amylase enzyme activity were further investigated (Shyam *et al.,* 2013).

2.5 Enzyme assay for amylase enzyme

Selected isolated from starch hydrolysis test were further sub-cultured on nutrient broth and were allowed to incubate for 48hr at 37°C. After incubation culture broths were centrifuged at 5000rpm for 10min at 4!. Cell free supernatant were recovered and used for enzyme activity by DNS spectrophotometric method. 1 mL of 1% starch in 2 mL, 0.1M phosphate buffer (pH 6.5) and 1 mL of diluted enzyme were incubated for 30 min at room temperature (37 °C). The reaction was arrested by adding 1 mL of DNS reagent and kept in a boiling water bath for 10 min and diluted with 8 mL of distilled water. The absorbance was measured at 540nm against blank prepared as above without incubation. One unit of α-amylase activity was defined as the amount of enzyme that liberates 1ìmole of reducing sugar (maltose equivalents) per minute under the assay conditions (U/min/mL of enzyme) (Shyam *et al,*. 2013).

Optimum temperature: The optimum temperature for amylase activities was determined by incubating amylase at different temperature (45-60 °C). 1 mL of substrate was taken into six different test tubes and 2 mL of phosphate buffer pH 7 was added in each test tubes. Tubes were marked with different temperature (at 4, 25, 37, 50, 75, 100°C). 1 mL of diluted enzyme solution was added in each tube.

Then tubes were incubated at specific temperature for 30 minutes. Reactions were terminated by adding 1 mL DNS reagent and the mixture incubated in boiling water for 10 min. After cooling at room temperature, the activity of enzymes were determined by taking the absorbance at 540nm and were expressed in U/min/mL of enzyme (Shankar *et al.,* 2011).

Optimum pH: The optimum pH for amylase activities was determined by incubating amylase at different temperature (5-9) 1% Starch was used as a substrate. Substrate solution was prepared in sodium phosphate buffer at pH 5, 6, 7, 8 and 9 in different test tubes. 1 mL each of diluted crude enzyme solution was added into buffer tubes. Then the mixture was incubated at room temperature for 30 min, reactions were terminated by adding 1 mL DNS reagent and the mixture was incubated in boiling water for 10 min. After cooling at room temperature, final volume was made to 12 mL with distilled water and the activity of enzymes was determined by taking the absorbance at 540nm and were expressed in U/min/mL of enzyme (Shankar *et al.,* 2011).

RESULTS

The purpose of the present study was to investigate the amylase enzyme activity of human microbiota consisting Human belly button, ear, nasal and armpits. Total 8 bacterial stains were isolated from the normal flora, among which 3 strains, i.e. J1, J2, & J3 Isolate were from Human belly button, where E1Isolate is from Ear, U1 and U3 from Nasal and S2 and S3 from Armpits. Morphological and Biochemical test were observed and they have revealed the presence of different starins of bacteria in Human belly button, ear, nasal and armpits as given in Table 1.

Molecular characterization of isolated were done by 16S rRNA gene sequencing and it has revealed the presence of *Enterobacter hormaechei* and *Bacillus mycoides* in Human belly button, where as *Bacillus thuringiensis* found to be present in the Ear. As far as Nasal cavity were concerned *Bacillus subtilis* and *Bacillus atrophaeus* found to be present by molecular charachterization, where as *Bacillus mycoides* and *Bacillus subtilis* found to be present in Armpits. There sequences have been submitted to NCBI-GenBank and their accession number has been achieved as given in Table 2. Phylogenetic tree was formed by using Phylogeny.fr **(**http://

Table 1. Morphological and biochemical characterization of Human microbiota

Morphological and Biochemical Test	J1	J2	J3	E1	U1	U3	S2	S3
Gram Staining	Negative	Positive	Negative	Positive	Negative	Negative	Positive	Positive
Shape	Rod	Rod	Rod	Rod	Rod	Cocci	Rod	Cocci
Methyl Red	Negative	Positive	Positive	Positive	Positive	Positive	Positive	-ve
Voges Proskauer	Negative	Negative	Positive	Positive	Negative	Negative	Negative	Positive
Citrate	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Lactose fermentation	Positive	Positive	Positive	Negative	Negative	Positive	Positive	Negative
Glucose Fermentation	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Negative
Catalase	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Starch Hydrolysis	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Positive
Bacteria Name	Enterobacter hormaechei	Enterobacter hormaechei	Bacillus mycoides	Bacillus thuringiensis	Bacillus subtilis	Bacillus atrophaeus	Bacillus mycoides	Bacillus subtilis

Table 2. Accession number of bacterial isolates

Sample Name	Isolation Source	Bacterial Name	Accession Number
J1	Human Belly Button	*Enterobacter hormaechei*	MK503501
J2	Human Belly Button	*Enterobacter hormaechei*	MK503447
J3	Human Belly Button	*Bacillus mycoides*	MK503615
E1	Ear	*Bacillus thuringiensis*	MK418792
U1	Nasal Cavity	*Bacillus subtilis*	MK503468
U3	Nasal Cavity	*Bacillus atrophaeus*	MK503495
S2	Armpit	*Bacillus mycoides*	MK503547
S3	Armpit	*Bacillus subtilis*	MK503496

www.phylogeny.fr/simple_phylogeny.cgi**)** (Figure 1-8). To screen amylolytic activity, starch hydrolysis test on starch agar plates was observed and among 8 bacterial strains only 4 bacterial strains it has shown positive response. Bacterial strain isolated from human belly button i.e. *Enterobacter hormaechei* (MK503501), *Enterobacter hormaechei* (MK503447) and *Bacillus mycoides* (MK503615) has shown amylase enzyme activity as given in Table 3. Maximum amylase enzyme activity was found to be

Table 3. Alpha amylase enzyme activity of Human Microbiota

Sample Name	Alpha amylase Enzyme Activity (U/min/mL of enzyme)
J1	0.701
J2	0.175
J3	6.66
S3	0.876

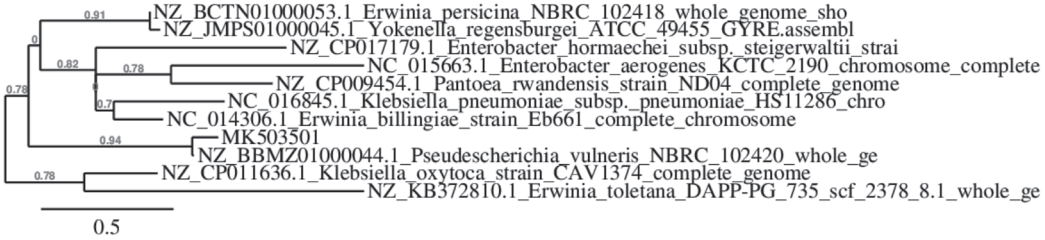


Fig. 1. Phylogenetic analysis of *Enterobacter hormaechei* (MK503501) by Neighbor Joining Method

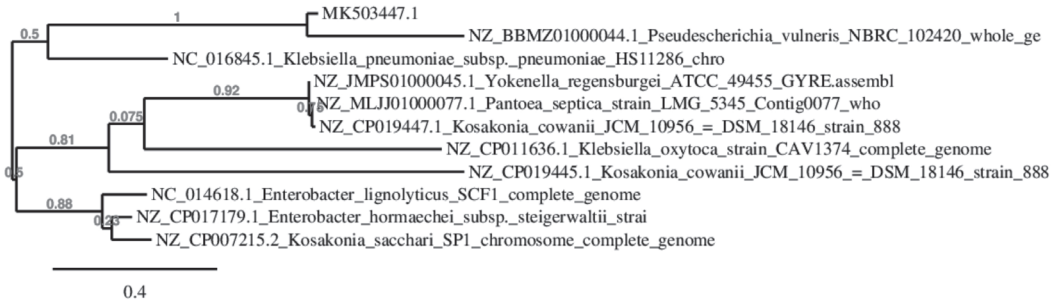


Fig. 2. Phylogenetic analysis of *Enterobacter hormaechei* (MK503447) by Neighbor Joining Method.

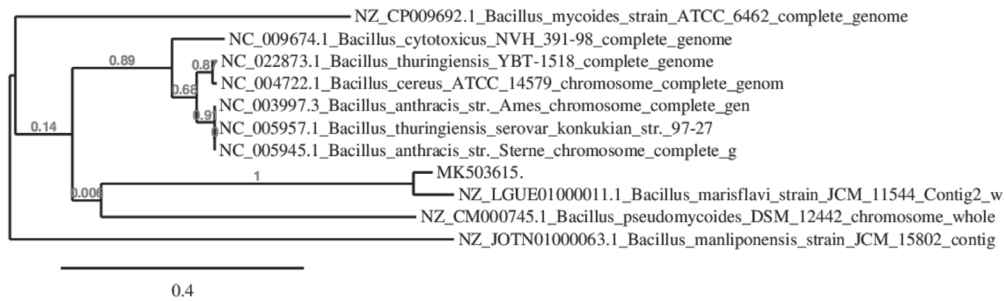


Fig. 3. Phylogenetic analysis of *Bacillus mycoides* (MK503615) by Neighbor Joining Method.

present in the *Bacillus mycoides* (MK503615), i.e. 6.66 U/min/mL of enzyme, where as lowest amylase enzyme activity was found to be present in *Enterobacter hormaechei* (MK503447), i.e. 0.175 U/min/mL of enzyme as given in Table 3. Temperature plays the significant role in the stability in enzyme activity. 25 °C was found to be optimum temperature of *Enterobacter hormaechei* (MK503501) and *Bacillus mycoides* (MK503615), where as *Enterobacter hormaechei* (MK503447) and *Bacillus subtilis* (MK503496) has shown 50 °C optimum temperature, respectively (Graph 1-4). pH of the growth medium plays an important role by inducing morphological changes in microbes and in

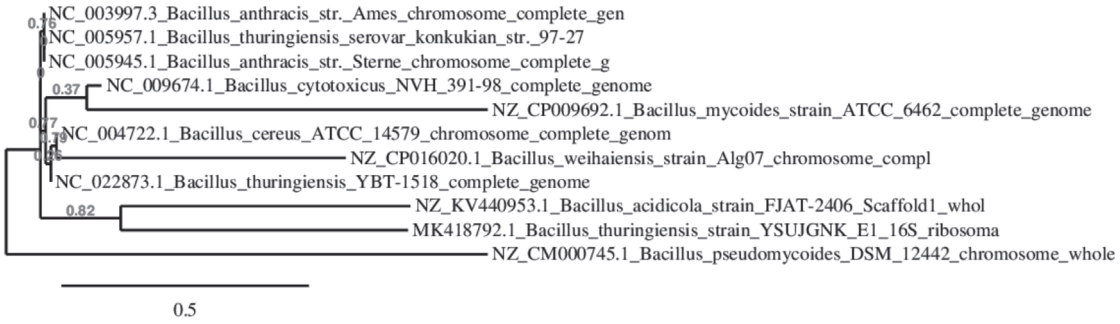


Fig. 4. Phylogenetic analysis of *Bacillus thuringiensis* (MK418792) by Neighbor Joining Method.

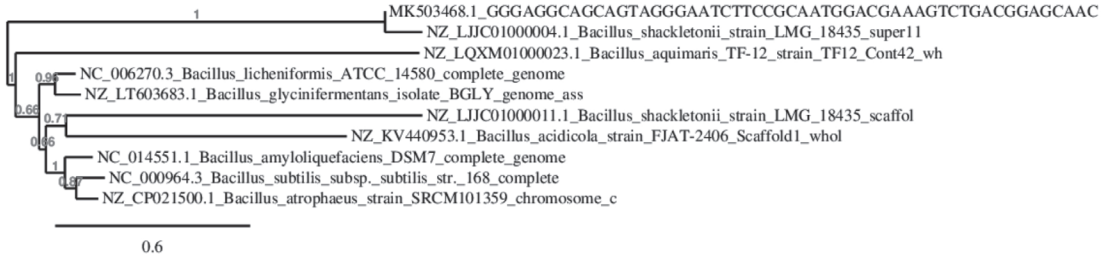


Fig. 5. Phylogenetic analysis of *Bacillus subtilis* (MK503468) by Neighbor Joining Method

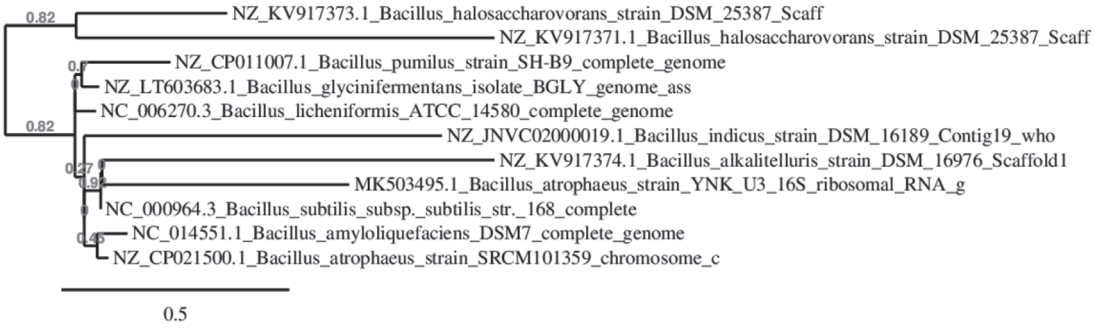


Fig. 6. Phylogenetic analysis of *Bacillus atrophaeus* (MK503495) by Neighbor Joining Method.

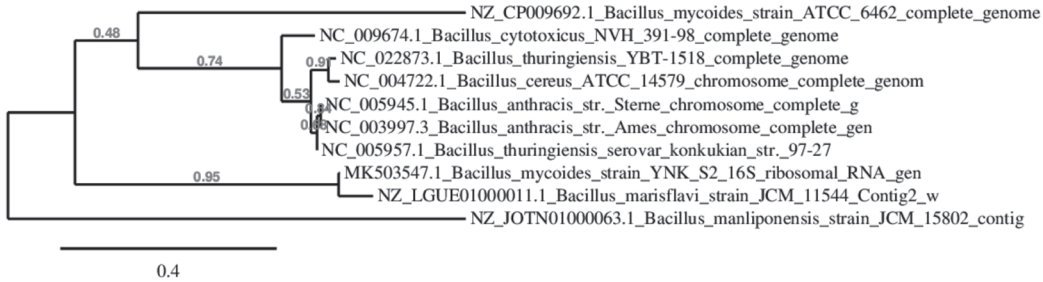


Fig. 7. Phylogenetic analysis of *Bacillus mycoides* (MK503547) by Neighbor Joining Method.

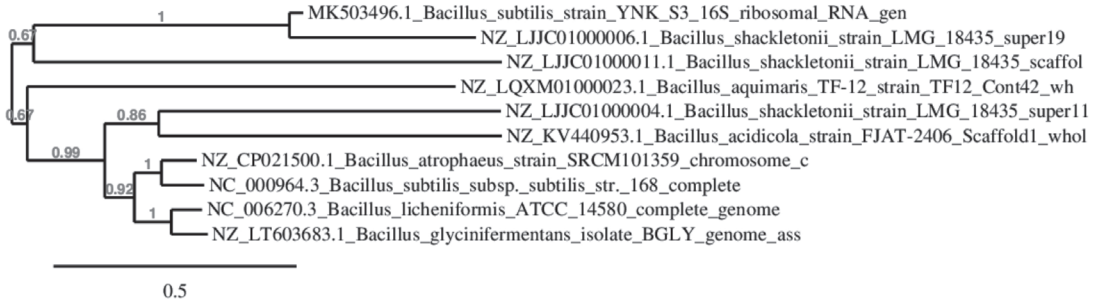
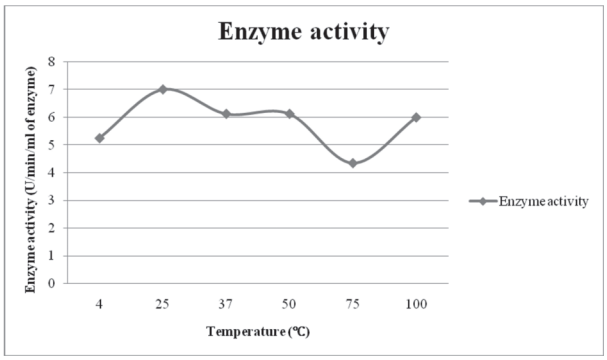
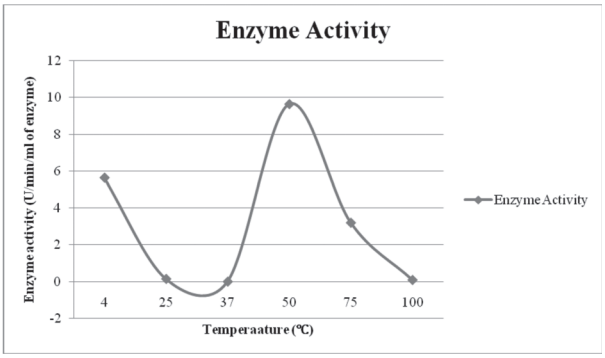


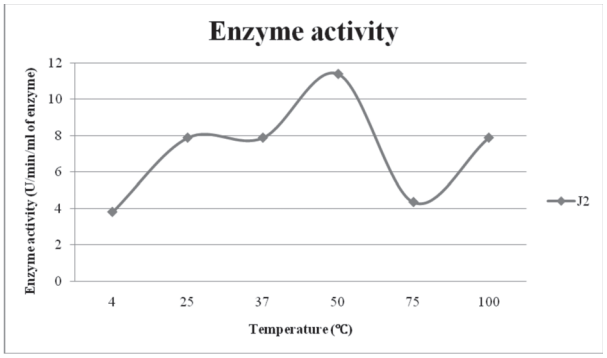
Fig. 8. Phylogenetic analysis of *Bacillus subtilis* (MK503496) by Neighbor Joining Method.



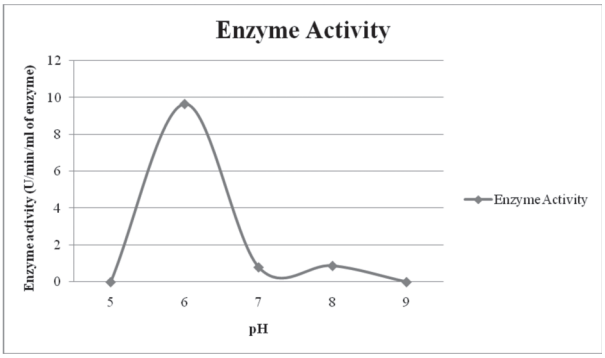
Graph 1. Effect of temperature on *Enterobacter hormaechei* (MK503501) amylase enzyme activity



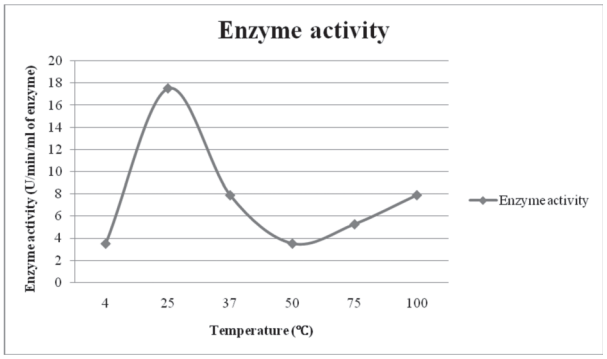
Graph 4. Effect of temperature on *Bacillus subtilis* (MK503496) amylase enzyme activity.



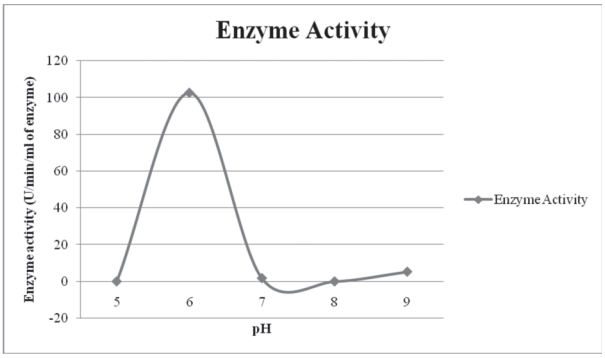
Graph 2. Effect of temperature on *Enterobacter hormaechei* (MK503447) amylase enzyme activity



Graph 5. Effect of pH on *Enterobacter hormaechei* (MK503501) amylase enzyme activity

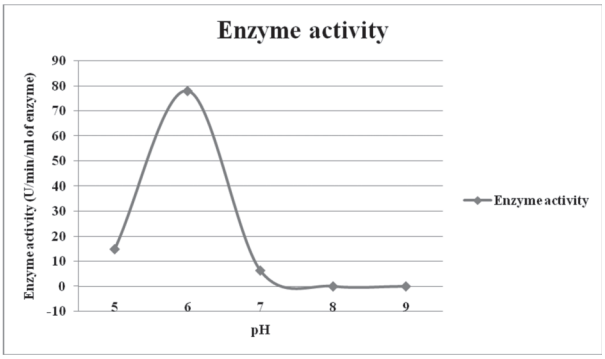


Graph 3. Effect of temperature on *Bacillus mycoides* (MK503615) amylase enzyme activity

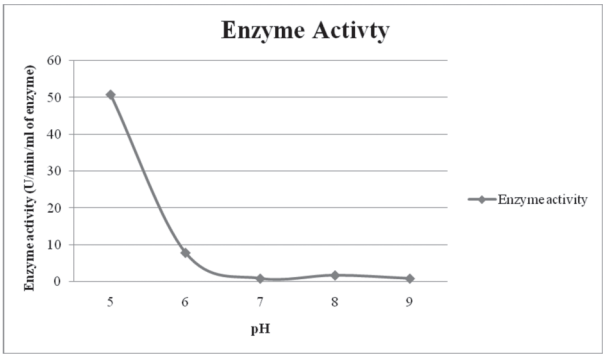


Graph 6. Effect of pH on *Enterobacter hormaechei* (MK503447) amylase enzyme activity

enzyme secretion. Changes in pH was observed during the growth of microbes that affect the product activity. As shown in Graph 5-7, *Enterobacter hormaechei* (MK503447), *Enterobacter hormaechei* (MK503501) and *Bacillus mycoides* (MK503615) was able to enzyme activity in pH range 5-7 and hence pH 6 found to be optimum pH. As far as the *Bacillus subtilis* (MK503496) was concerned, it has shown optimum pH at 5 that reveal its maximum amylase enzyme activity as shown in Graph 8.



Graph 7. Effect of pH on *Bacillus mycoides* (MK503615) amylase enzyme activity



Graph 8. Effect of pH on *Bacillus subtilis* (MK503496) amylase enzyme activity.

DISCUSSION

Microbial consortium is defined as the species diversity that drastically changes depending upon the surrounding environmental conditions. Human beings have evolved throughout with the microbial community since decades but still lacks the information regarding species composition and functions (Alivisatos *et al.,* 2015). A known microbial community has been utilized for human welfare in two stages, i.e. naturally mixed culture and pure culture. The pure culture thus, obtained by the researchers can be untiled as a single strain that has its own morphological, physiological, biochemical, and genetic characteristics. Pure culture nowadays, are being utilized in biochemical engineering and modern biotechnology. The best example of utilization of pure culture from biotechnological background comes from the products being synthesized at the industrial level which includes amino acids, organic acids, antibiotics, and enzymes. To date, many bulk biotechnological products such as amino acids, organic acids, antibiotics, and enzymes are almost produced by pure cultures. α-amylase are exoenzymes that catalyzes the hydrolysis of internal α-1, 4-glycosidic linkages in starch in low molecular weight product and such as glucose, maltose, and maltotroise units. Amylase are among the most important enzymes that are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 30% of the world enzymes market (Behal *et al.,* 2006). The present study deals with the investigation for production of alpha amylase enzyme from normal bacterial flora, i.e. human microbiota. The following study is an initial screening in the field of microbial activity and enzyme production to use the normal flora of human body for the industrial purposes. From the present study, it was found that *Enterobacter* sp. and *Bacillus* sp. was the most prominent sp present on the Human belly button, ear, nasal and armpits of, whereas previous study has shown the presence of *S. epidermidis* as commonest bacterial isolate human ear canal (Prasanna *et al.,* 2015). Frequently encountered genera in the year 1975 study revealed the presence of *Streptomyces*, and *Enterobacter* in the belly button samples, where as *Enterobacter hormaechei* (MK503501 and MK503447) and *Bacillus mycoides* (MK503615) were found to be present in the above study. In a recent study, *Bacillus subtilis* (MK503468) and *Bacillus atrophaeus* (MK503495) were found to be present in the nasal cavity where as compared to previous study, *Streptococcus thoraltensis* and several others were found to be present in the nasal cavity and the oropharynx (AlWakeel *et al.,* 2017). As far as the alpha amylase enzyme production was concerned *Enterobacter hormaechei* (MK503501 and MK503447), *Bacillus mycoides* (MK503615) and *Bacillus subtilis* (MK503496) has shown the enzyme producution where as *B. subtilis*, has shown enzyme production, i.e. 858.6±41.9 U/ mg was obtained in a medium containing 0.5% cotton stalk in 72 h (Akcan *et al.,* 2011). Previous studies has revealed thermostable amylase producing *Bacillus species* revealed an optimum enzyme activity at pH 8,

whereas compared to present study *Enterobacter hormaechei* (MK503447), *Enterobacter hormaechei* (MK503501) & *Bacillus mycoides* (MK503615) has shown enzyme activity between pH range 5-7 and hence pH 6 found to be optimum pH. α-amylase enzyme activity also varies with temperature with respect to the previous study *Bacillus* sp. showed the amylase activity at different temperature from 35 to 45 °C has gradual decrease from 0.992 U/mL to 0.545 U/mL, where as compared to present study 25 °C was found to be optimum temperature of *Enterobacter hormaechei* (MK503501) and *Bacillus mycoides* (MK503615), where as *Enterobacter hormaechei* (MK503447) and *Bacillus subtilis* (MK503496) has shown 50 °C optimum temperature, respectively (Graph 1-4).

CONCLUSION

From the present study, authors would like to conclude that bacteria which has been isolated from Human belly button, ear, nasal and armpits has revealed the load of both Gram negative bacteria (*Enterobacter hormaechei)* and Gram positive bacteria (*Bacillus mycoides, Bacillus subtilis, Bacillus thuringiensis & Bacillus atrophaeus)*. Amylase enzyme production was found to be in *Enterobacter hormaechei*, *Bacillus mycoides* and, *Bacillus subtilis.* This present study suggests to use normal flora of human body for the industrial purposes and further to check the pharmaceutical properties like antioxidant and bacteriocin production for future purposes.

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