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Original Article

Extraction of chitin from edible crab shells of *Callinectes sapidus* and comparison with market purchased chitin

Extração de quitina de cascas de caranguejo comestíveis de *Callinectes sapidus* e comparação com quitina adquirida no mercado

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Abstract

Chitin and its derived products have immense economic value due to their vital role in various biological activities as well as biomedical and industrial application. Insects, microorganism and crustaceans are the main supply of chitin but the crustaceans shell like shrimp, krill, lobsters and crabs are the main commercial sources. Chitin content of an individual varies depending on the structures possessing the polymer and the species. In this study edible crabs' shells (*Callinectes sapidus*) were demineralized and deproteinized resulting in 13.8% (dry weight) chitin recovery from chitin wastes. FTIR and XRD analyses of the experimental crude as well as purified chitins revealed that both were much comparable to the commercially purchased controls. The acid pretreatment ceded 54g of colloidal chitin that resulted in 1080% of the crude chitin. The colloidal chitin was exploited for isolation of eighty five chitinolytic bacterial isolates from different sources. Zone of clearance was displayed by the thirty five isolates (41.17%) succeeding their growth at pH 7 on colloidal chitin agar medium. Maximum chitinolytic activity i.e. 301.55 U/ml was exhibited by isolate JF70 when cultivated in extracted chitin containing both carbon and nitrogen. The study showed wastes of blue crabs can be utilized for extraction of chitin and isolation of chitinolytic bacteria that can be used to degrade chitin waste, resolve environmental pollution as well as industrial purpose.

Keywords: crab shells, chitin, chitinases, chitinolytic bacteria.

Resumo

A quitina e seus produtos derivados têm imenso valor econômico devido ao seu papel vital em várias atividades biológicas, bem como em aplicações biomédicas e industriais. Insetos, microrganismos e crustáceos são o principal suprimento de quitina, mas a casca dos crustáceos como camarão, krill, lagosta e caranguejo são as principais fontes comerciais. O conteúdo de quitina de um indivíduo varia dependendo das estruturas que possuem o polímero e da espécie. Neste estudo, as cascas de caranguejos comestíveis (*Callinectes sapidus*) foram desmineralizadas e desproteinizadas, resultando em 13,8% (peso seco) de recuperação de quitina a partir de resíduos de quitina. As análises de FTIR e XRD do bruto experimental, bem como das quitinas purificadas, revelaram que ambas eram muito comparáveis aos controles adquiridos comercialmente. O pré-tratamento com ácido cedeu 54 g de quitina coloidal que resultou em 1.080% da quitina bruta. A quitina coloidal foi analisada para isolamento de 85 isolados bacterianos quitinolíticos de diferentes fontes. A zona de eliminação foi exibida pelos 35 isolados (41,17%) que sucederam seu crescimento a pH 7 em meio de ágar de quitina coloidal. A atividade quitinolítica máxima, ou seja, 301,55 U / ml, foi exibida pelo isolado JF70 quando cultivado em quitina extraída contendo carbono e nitrogênio. O estudo mostrou que resíduos de caranguejos azuis podem ser utilizados para extração de quitina e isolamento de bactérias quitinolíticas que podem ser usadas para degradar resíduos de quitina, resolver a poluição ambiental e também para fins industriais.

Palavras-chave: caranguejo, quitina, quitinases, bactéria quitinolítica.

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1. Introduction

Chitin is the most copious renewable natural polymer that assembled in marine invertebrates, insect's exoskeleton, cell walls of fungi and algae. Aquatic products which constitute of organisms loaded in chitinous matter are approximately 10% of total global landing and about 10^{12} tons of chitin wastes accumulates in ocean every year (Jahangiri et al., 2019; Ray et al., 2019; Sun et al., 2019). Ecdysis (shedding of cuticle) as well as senescence is the dynamic processes that consequent in unremitting hail of chitin to the ocean base which is recognized as marine snow, while there is no quantitatively significant accretion of chitin in sediments of ocean due to its efficient degradation and metabolization by bacteria (Elsoud and El Kady, 2019; Rameshthangam et al., 2018).

Among sea food the main commercial sources of chitin are crustacean shells due to their high content and ready availability (Ray et al., 2019; Xu et al., 2020). Crabs are being used in large quantity, producing considerable amount of their shell waste into the environment which can be utilized for chitin recovery and production of chitinases for degradation of heaps of chitin wastes. Chitinases can be constructed towards various valuable applications including biological control of pathogenic fungi (Liu et al., 2019; Loc et al., 2019) and harmful insects (Ray et al., 2019), production of single cell protein, production of biofuel, eradication of malaria and various application in food, agriculture, pharmaceuticals and chemical industries (Ali et al., 2020; Asif et al., 2019; Oyeleye and Normi, 2018).

Chitin is a linear polymer of N-acetylglucosamine with β (1-4) linkage and is insoluble in water. Chitin and its derived products has attracted a great attention and marketing power due to their possible applications in biotechnology including medicine, cosmetics, pharmacology, agriculture, biological control and wastewater treatment. Non-antigenicity, bio-compatibility, bio-degradability and non-toxicity are the useful biological activities which have been exhibited by chitin and its derived products. Recently they have displayed a high value-added application that is why gaining the interest of many investigators and made them curious for the new promising sources of chitin (Asif et al., 2019; Kumar and Zhang, 2019).

Several techniques have been proposed and used up till now for the chitin extraction from its diverse sources. Many of them depend upon the chemical procedures which involve the removal of protein and elimination of inorganic matter (demineralization). Some of them embrace the removal of pigment from extracted chitin which improves its color, by using chemical oxidation and solvent extraction method (Beaney et al., 2005; Yadav et al., 2019). For the chitin fabrication on commercial scale from crustaceans the conventional methods available are the removal of protein (deproteination) with alkali at high temperatures and mechanical grinding (demineralization) with strong acids (Pighinelli et al., 2019; Thirunavukkarasu and Shanmugam, 2009).

In this study we aimed to extract the chitin from edible blue crab and compared with market available purified chitin to exploit it to isolate chitinolytic bacteria. The exploitation of crab shell waste by potent bacteria would be projected not only to solve the issue of waste generation but also environmental problems.

2. Materials and Methods

2.1. Ethical statement

The study was conducted in accordance with declaration of University of the Punjab and the protocol was approved by Ethics committee of the said University.

2.2. Crab collection

Blue crab (*Callinectes sapidus)* was purchased from local sea food shop, washed with simple tap water and then meat was separated from the shell. The shells were dehydrated in oven at 105°C till its weight became constant then grinded to powder.

2.3. Demineralization and deproteinization of crab shell

Crab shells were treated according to the process given in (Jabeen and Qazi, 2014). For demineralization 5 g of shell powder was added in 0.55M HCl (45 ml) for 2 h at room temperature. After that for deproteinization 0.3 M sodium hydroxide (100ml) was added repeatedly for 1 h at 80°C. Now the treated suspension was filtered and washed with distilled water two to three times. The powder on filter paper mainly containing chitin was dried and saved in dried bottle.

2.4. Characterization of chitin

2.4.1. Fourier Transmission Infra Red spectroscopy (FTIR)

Treated crab shells and market purchased chitin were analyzed on FTIR system. KBr pellets were used to prepare samples with 2:100 (w/w) i.e 2%. Absorbance was taken with the resolution of 2 cm^{-1} and scan 4.

2.4.2. X.Ray Diffraction (XRD)

Treated crab shells and market purchased chitin were analyzed on X-ray diffractograms and observed with Cu–Kα (40 kV and 40 mA) radiation with graphite chromators at 298 K. The relative intensity was recorded in a 10 – 85°dispersion range (2θ).

2.4.3. Processing for colloidal chitin

Colloidal chitin was achieved by technique given in (Jabeen and Qazi, 2014). Concentrated Hydrochloric acid (60ml) was added in 5 g chitin powder with vigorous shaking for 1 h and filtered through glass wool. The filtrate was treated with 200 ml of 50% ethanol with continuous shaking. The colloidal chitin was filtered through filter paper and washed repeatedly with distilled water until the spent water became neutral. Colloidal chitin was separated from the filter paper, weighed and stored in brown bottle at 4°C.

2.4.4. Isolation of chitinolytic bacteria

Eighty five bacterial isolates were separated from different samples of soils containing insects, their mound and nearby areas of their dwellings. Samples were run on 1% colloidal chitin containing selective medium. Colony forming units (C.F.U.) in the samples were also counted on selective medium. Zone producing isolates were pure cultured and preserved on glycerol stocks.

2.4.5. Estimation of chitinolytic activity

Chitinolytic activity was assessed by reducing sugars released from the chitin following method of Sadafi et al. (Sadfi et al., 2001). The standard curve was plotted with N-acetylglucosamine (NAG). One unit of chitinolytic activity was described as 1 micromole of GlcNAc per mg of protein per minute.

3. Results and Discussion

3.1. Processing of chitin containing waste

Commercially crab and shrimp shells are being used as chitin source for their availability and easy access. (Gadgey and Bahekar, 2017; Yadav et al., 2019). Jabeen and Qazi (Jabeen and Qazi, 2014) reported that chitin is most renewable resource as tons of chitin is produced every year on earth. The utilization of insect waste while solving an environmental problem will decrease the production costs of microbial chitinases. Because of hydrophobic property, its degradation is not easily possible due to its inert behavior but it has the potential for bioconversion to monomers (GluNAc) and chito-oligosaccharides by enzyme-catalyzed reactions (Jung and Park, 2014; Schmitz et al., 2019).

Chitin was used as only carbon source for chitinases production which has abundantly produced by food industries. Different chemical and biological pretreatments methods to enhance production have been reported. In the present study blue crabs shells were cleaned, mashed and ground, then demineralization and deproteinization was carried out for removal of minerals and proteins respectively. After processing and dehydraion 0.55g chitin was obtained that recovered 13.8% of the total crab shell waste. These results are in accordance with the work of many workers. Pandharipande and Bhagat (2016) reported the yield of chitin between 10.60-12.73%, extracted from crab shells. Narudin et al. (2020) also declared that from crab 11.73% chitin content was achievable. Crabs and its shells used are shown in Figure 1.

Colloidal chitin was prepared with acid pretreatment for easy utilization of chitinolytic bacteria. Chitin after acid treatment yielded 54g of colloidal chitin which was 1080% of the crude chitin. Song et al. (2020) reported that colloidal chitin was utilized more rapidly than crude chitin. The specific method thus adapted in this study for preparing colloidal chitin capitulate slightly higher colloidal than other methods examined by various workers. This method is also relatively quicker than many others and the product is easy to be uptaken by bacteria. While estimating the chitin utilization rate, Seki(Seki, 1965) found that 10^{10} bacterial cells in 1 cm^3 of the soil, could decompose about 30 mg each day at 25°C. He also estimated that chitin in ocean gets decomposed within 140 days at 15°C and required 370 days at 5°C, whereas below 5°C it acquire 500 or more days.

3.2. XRD and FTIR analysis of chitin

XRD analysis of the commercial chitin revealed a difference of peak around 25 angle which was present only in case of Roth. The sigma chitin had an additional peak

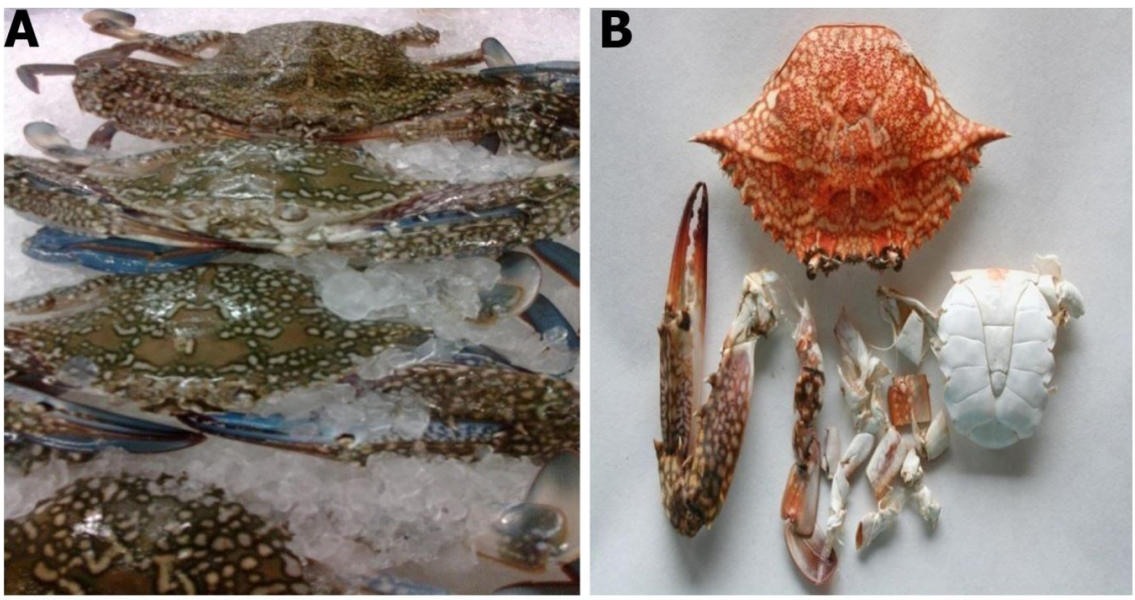

Figure 1. Edible blue crabs (*Callinectus sapidus*) (A) and parts used for the isolation of chitin polymer (B).

around 50 angle representing the last plateau (Figure 2). The crude chitin had a very close general look with the Sigma, expecting having an additional peak around 29 angle and higher intensity of the peak at 43 angle. However, the purified chitin had a mimic pan of XRD analysis with that of Sigma chitin (Figure 2).

A sharp peak is appeared in sigma, Roth and purified blue crab chitin near 19°, not found in crude shell and weak peaks after 20°. In other studies, XRD peaks of α-chitin observed from various organisms i.e. shrimp, crab, krill, anthozoa, and insects sharply peak around 12°, with weak peaks around 19, 23 and 26° (Wang et al., 2013). These results showed that shell of *C. sapidus* is mainly consisted of α-chitin.

FTIR analyses of the two commercially purified chitin i.e. Sigma and Roth revealed small differences in the percent transmittance upto wave number 1800, there after the detailed differences became prominent for a very short segment of wave number. An overview throughout wave spectra from 390 upto 1100, however, depicted a comparable trend (Figure 3). The crude as well as purified chitin prepared during course of this study had quite much comparable patterns of the percent transmittances with more prominent vertical oscillation in case of purified polymer (Figure 3). The chitins extracted and purified in this study resemble more closely in term of % transmittance to that of Roth as compare to Sigma. FTIR patterns showed the bands all corresponds to stretching and vibration of O-H, N-H and CO bonds as given in Table 1.

3.3. Isolation and screening of chitinolytic bacteria.

Although chitinase producing organism are wide spread in nature, microbes have been exploited as preferred source because of their rapid enzymes production, limited requirement of cultivation space and their easy enzyme extraction protocols from fermented broth (Gupta et al., 2017). Chitinase production is found in many microorganisms; and among them large numbers of bacterial species is known to produce chitinases. Considering the richness of microbial diversity, there is always a chance of new variety carrying better enzymatic character and their suitability for commercial exploitation always exists.

Eighty five bacterial isolates were separated from samples comprising insects, their mounds and their affected fields. Samples were processed on selective agar medium

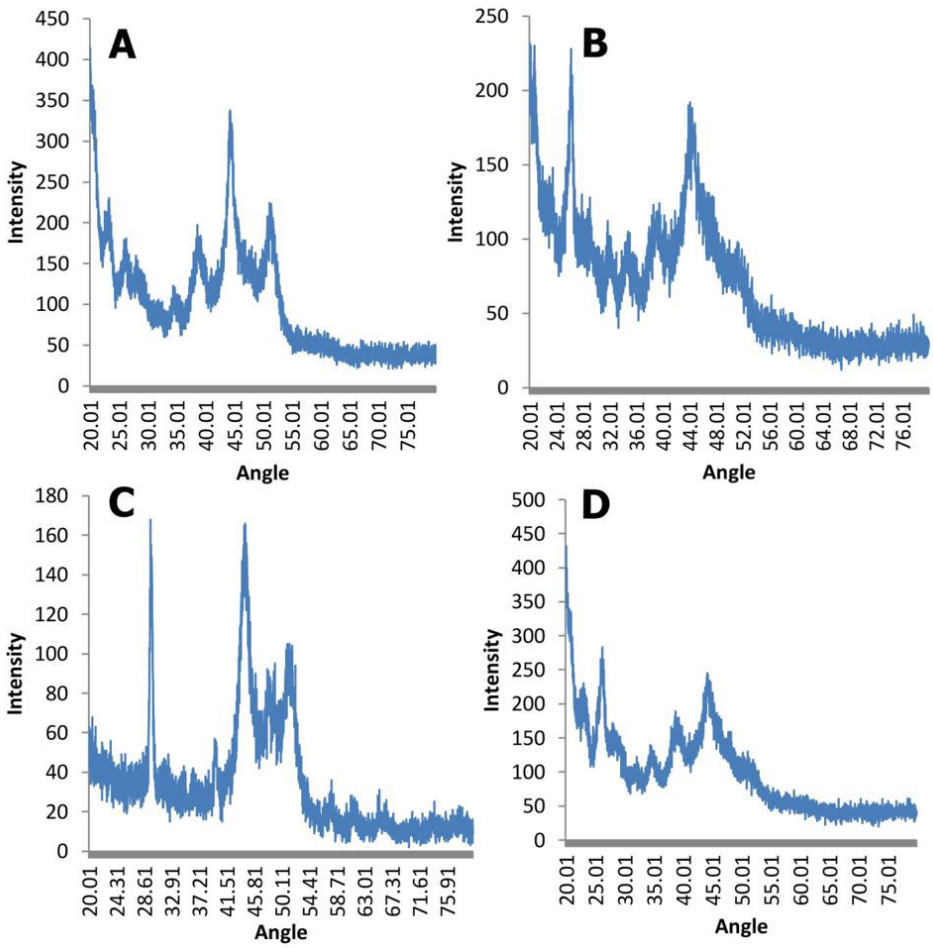

Figure 2. XRD spectra of Sigma (A); Roth (B); blue crab (*Callinectes sapidus*) crude (C); and purified chitins (D).

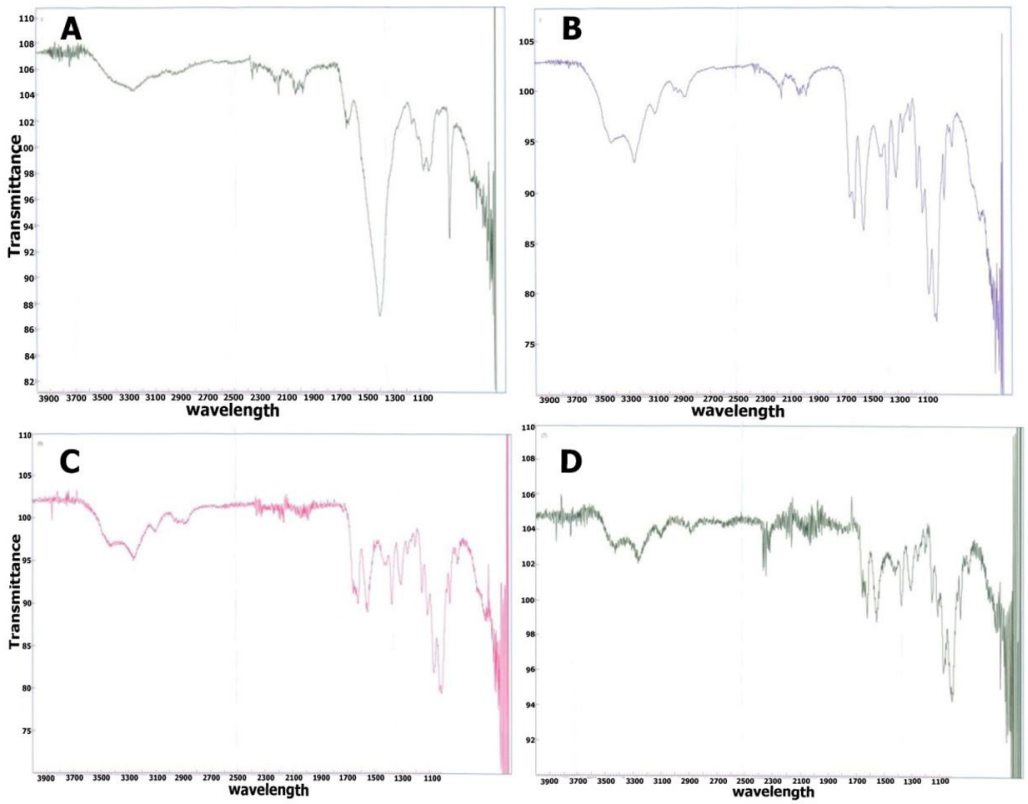

Figure 3. FTIR analysis of Sigma (A); Roth (B); Blue crab (*Callinectes sapidus*) crude (C); and Purified chitins (D).

Table 1. The FT-IR bands (cm^{-1}) of other commercial chitin (Kaya et al., 2015).

Functional group and vibration modes	Classification	Other commercial chitin
O–H stretching	—	3437
N–H stretching	—	3101–3259
CH_3 symmetrical stretch and CH_2 asymmetric stretch	Aliphre2watic compounds	2937
CH_3 symmetrical stretch	Aliphatic compound	2867
C–O secondary amide stretch	Amide I	1654
C–O secondary amide stretch	Amide I	1620
N–H bend, C–N stretch	Amide II	1553
CH_2 ending and CH_3 deformation	—	1430
CH bends CH_3 symmetrical deformation	—	1376
CH_2 wagging	Amide III, components of protein	1318
Asymmetric bridge oxygen stretching	—	1155
Asymmetric in-phase ring stretching mode	—	1114
C–O–C asymmetric stretch in phase ring	Saccharide rings	1068
C–O asymmetric stretch in phase ring	—	1024
CH_3 wagging	Along chain	952
CH ring stretching	Saccharide rings	896

containing chitin the only carbon source. Among isolates bacterial 35 exhibited vivid hydrolysis zone at pH 6.0. Maximum ZS to CS ratio was 5.0 mm (Table 2).

Based upon the chitin hydrolysis zones thirty bacterial isolates were selected for further study. The larger and clearer the zone of chitin hydrolysis, the more efficient the bacterial isolate was considered. The method was considered as primary quantitative test for confirmation of chitinolytic bacteria. The ratio of the zone size and colony size indicates the extent of chitinolytic exoenzymes diffusibility. It represents simple and inexpensive method for the isolation of chitinolytic bacteria from pool of bacterial diversity.

Table 2. chitin hydrolysis, growth and chitinase activity on chitin medium following 5 days of growth at 37°C.

Isolate code	Ratio of Zone size to Colony size (ZS/CS)	C.F.U./ml x 10^6	Chitinase activity (U/ml)
JF-1	3.5	197	225.375
JF-9	2.0	92	271.334
JF-10	1.76	291	173.447
JF-14	2.66	1.52	295.112
JF-16	2.0	0.44	281.334
JF-20	2.8	134	200.516
JF-24	1.8	93	64.849
JF-25	2.05	0.91	73.495
JF-27	4.0	1.31	143.749
JF-28	5.0	96	179.416
JF-35	1.3	1.14	162.318
JF-38	2.25	1.73	181.061
JF-41	2.22	1.36	79.980
JF-42	1.75	1.92	62.687
JF-47	4.5	1.11	162.123
JF-50	2.66	1.51	95.112
JF-55	3.5	1.18	125.375
JF-57	4.0	2.19	143.749
JF-59	1.5	159	174.041
JF-60	3.0	96	108.082
JF-62	5.0	58	199.416
JF-65	3.0	1.52	145.437
JF-66	3.3	76	216.991
JF-68	2.1	156	230.214
JF-70	2.9	1.23	301.548
JF-81	0.8	143	231.295
JF-82	1.2	245	112.174
JF-83	1.2	2.76	98.324
JF-84	0.8	3.4	45.78
JF-85	1.25	7.9	117.651

From 85 isolates only 27 isolates (35.29%) showed vivid zone of chitin hydrolysis. Korany et al (Korany et al., 2019) reported that among the thirty four isolates only four isolates produced zones of clearance (hydrolysis) on chitin agar medium. Similarly Ajayi et al. (2016) had isolated 36 chitinolytic isolates but selected 24 on the basis of highest chitnolytic index. Since chitinases are able to diffuse through agar, methods to identify chitinolytic bacteria are generally based on monitoring the hydrolysis of chitin polymer incorporated into agar medium (Hardoko et al., 2020).

Further twelve isolates were selected on the basis of chitinolytic activity units shown in Table 2. Among isolates JF70 yielded maximum chitinase activity (301.55 U/ml). This was followed by the isolate JF14 (295.11 U/ml) and the least producing chitinases was JF 59 (174..04 U/ml) shown in Table 2 following five days of submerged fermentation. All the twelve isolates were mesophilic and thermostable exposed their ability to work in harsh environmental conditions. Chitin being the second most abundant carbohydrate on the earth, provides richness in environment suitable for their survival and propagation of the chitinolytic microorganisms. Thus, the copious number of bacterial isolates being reported here and the previous studies is not surprising. Organisms containing chitin in their structure like fungi and insects produce chitinases for their growth and development purpose but bacteria produce chitinases in their saprophytic phase only to get carbon and nitrogen from chitin polymer (Lacombe-Harvey et al., 2018; Veliz et al., 2017). In the present study 71.42% of the samples represented soil's different nature. Soil bacteria are excellent sources of chitinolytic enzymes and could be use preferably for catabolic conversion of chitinous waste into useful products for diverse applications in biotechnology, medicine and agriculture (Schmitz et al., 2019; Sunny et al., 2018).

4. Conclusion

This is the first study to report comparison in the chitin extracted from blue edible crab and market purchased chitin. Characterization of chitin was carried out with FT-IR and XRD analysis. It is concluded that purified chitin of analytical grade is comparable to the Sigma and Roth brand, can be prepared from chitin wastes collected from edible blue crabs heaps in sea food markets by chemical methods which can not only provide purified chitin but also solve the environmental problems by converting the chitin to useful purpose for production of chitinolytic bacteria and various useful products can be exploited for diverse industrial purposes.

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