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Co-production of hydrolytic enzymes using gram waste and its optimization through the OVAT approach for *Nesterenkonia sp.* K-15-9-6

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ABSTRACT

Agro-industrial and food processing wastes need proper treatment if sustainability is to be achieved. This study proposes a systematic approach to convert gram waste into valuable products (hydrolytic enzymes) via submerged fermentation (SmF) using *Nesterenkonia* sp. K-15-9-6. The co-production approach, involving the synthesis of multiple enzymes in a single medium, offers efficiency gains with cost reduction. To enhance the yield of enzymes, the key parameters wereoptimized, including incubation period (24-120 h), pH (7.0-11.0), gram waste concentration (2.0-10.0g%), inoculum volume (3.0-15.0%v/v), medium volume and NaCl (0.0-15.0%w/v). The research finding indicated that incubation time, 48h; pH, 9.0; gram waste, 8g%; inoculum volume, 5%v/v; medium volume, 75mL and NaCl concentration, 2.5%w/v ere optimum for co-production of hydrolytic enzymes.The maximum protease (129 U/mL), xylanase (5.12 U/mL), cellulose (5.66 U/mL), amylase (5.89 U/mL), and lipase (4.99 U/mL) production was observed under optimized conditions.In summary, this work highlights the potential of *Nesterenkonia* sp. K-15-9-6 for co-producing hydrolytic enzymes and sustainable managing of gram waste.

Keywords : Gram waste, Co-production, Hydrolytic enzymes, *Nesterenkonia*, Sustainable Waste Management

INTRODUCTION

The demand of biocatalysts has been significantly increased in recent years, prompting researchers to explore innovative and cost-effective approaches for production of biocatalyst (Sharma *et al.*, 2022). Co-production, a strategy involving the simultaneous synthesis of multiple enzymes, emerges as a promising avenue for enhancing production yield and reducing production cost (Kaur *et al.*, 2020). An intriguing method entails the utilization of gram waste as an economical substrate for the co-production of hydrolytic enzymes. Gram waste, encompassing residues from gram crops like chickpeas, husks, lentils, peas, and other by-products, plays a substantial role in global agriculture and serves as a considerable biomass resource for the biosynthesis of various biomolecules (Prakasham *et al.*, 2006). Gram waste offers several advantages as a substrate, being abundant, cost-effective, and displaying diverse biochemical compositions conducive to the growth of enzyme-producing microorganisms. Moreover, the nutritional content of gram waste fosters an

environment favorable for the synthesis of hydrolytic enzymes, making it an ideal candidate for co-production processes. This waste not only presents environmental challenges of safe disposal but also offers a sustainable solution via biosynthesis of multiple hydrolytic enzymes (Rodríguez Couto, 2008; Suganthi *et al.*, 2011).

Usually, enzyme production has relied on conventional sources such as microorganisms, plants, and animals. Among the different group of bacteria, halophilic bacteria have adapted to thrive and function optimally in conditions with high salt concentrations (Raval *et al.*, 2014; Gunde-Cimerman et al., 2018; Raval *et al.*, 2022). The combination of halophilic bacteria and agro-food waste in submerged fermentation for enzyme production presents a synergistic approach aligning with cost-effectiveness, reduced contamination risk, and the sustainable utilization of waste materials. This approach holds implications for various industries, including biotechnology, food processing, and waste management (Sharma *et al.*, 2023).

Conversely, traditional methods often encounter challenges related to high production costs, limited scalability, and environmental impact. The pursuit of sustainable alternatives has intensified the exploration of unconventional substrates, leading researchers to investigate agro-industrial residues (Arya *et al.*, 2022a). However, co-production offering distinct advantages. Firstly, it maximizes resource utilization, ensuring a more efficient and sustainable process. Secondly, co-production can result in synergistic effects, where the presence of one enzyme enhances the production of others, creating a cascading effect that boosts overall productivity (Rathore and Singh, 2021; de Siqueira and Öner, 2023). By leveraging the synergies between different enzymes, co-production aligns with the principles of green chemistry, emphasizing resource efficiency and reduced waste. Furthermore, the co-production of hydrolytic enzymes using gram waste as a substrate represents a promising frontier in enzyme biotechnology.

MATERIALS AND METHODS

Microorganism

Nesterenkonia sp. K-15-9-6, isolated from the Gulf of Khambhat, Gujarat, India was previously identified by molecular approaches. The strain was previously studied for hydrolytic enzyme production, dye degradation, and production of plant growth-promoting substances. This isolate was identified based on 16S rRNA sequence and submitted to NCBI Gene bank with the accession number ON331909. The bacterium was maintained on a modified nutrient agar medium (peptone 10 g/L, yeast extract 5 g/L, NaCl 25 g/L, agar 30 g/L,and pH 9.0) at 5 °C for further study.

Inoculum preparation

The K-15-9-6 culture from the slant was inoculated in sterile nutrient broth (pH 9.0) supplemented with 2.5% (w/v) NaCl and incubated at 37°C for 24 h. Growth was measured at 600nm and set the optical density of 1.0 which corresponded to 1.0% (v/v) inoculum. The prepared culture suspension was used as a 3.0% (v/v) inoculum for the experiment.

Co-production of hydrolytic enzymes

Submerged Fermentation (SmF) was carried out in 250 mL Erlenmeyer flasks containing 3 g of each dry solid agro-food waste separately. The flasks were autoclaved at 121 °C for 15 min and then, the corresponding amount of basal solution was inoculated with seed culture of 3% (v/v) and

was mixed with solid substrates. The basal solution contained 3% (w/v) gelatin, 2.5% (w/v) NaCl, and 1.0% (w/v) peptone. pH was adjusted to 9 by 20% Na_2CO_3. The inoculated flasks were incubated at 37 °C for 120 h, on a 120 rpm rotary shaker. After the fermentation period, the broth was filtered using a muslin cloth, and the liquid fraction was collected manually and centrifuged for 10 min at 10,000 rpm. The pellets were discarded from the supernatants and used as crude enzymes for further analysis.

Quantitative assay for hydrolytic enzymes

The activity of xylanase, cellulase, and amylase was assayed by modifying the method of Bailey et al. (1992) by measuring the amount of reducing sugars liberated from the substrate using the DNS method (1959). Reaction mixture containing 3 mL of 0.5% birch wood xylan, 1% CMC, and 1% starch as substrate respectively for xylanase, cellulase, and amylase (prepared in 20 mM glycine-NaOH buffer, pH 9.0) and 1 mL of crude enzyme. The absorbance was measured at 540 nm in a spectrophotometer. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of product (glucose/ xylose) released/min/mL (U/mL) under the assay conditions.

The lipase activity was estimated by modifying the method described by Maldonado et al (2012). The reaction mixture consisted of 19 mL of olive oil/Arabic gum emulsion (5% olive oil and 5% Arabic gum) in 100 mM potassium phosphate buffer, pH 7.0, and 1 mL of crude enzyme. One unit of lipase activity was defined as the amount of enzyme that liberates 1micromole of fatty acids equivalent per minute under the assay conditions.

Anson-Hagihara method (1958) was used to analyze protease production with Hammerstein casein as a substrate. In this assay, the cell-free supernatant (0.5 ml) was incubated for 30 min at 37 °C with 3.0 ml of 0.6% (w/v) casein (prepared in 20 mM glycine-NaOH buffer pH 9.0). A UV-Vis spectrophotometer was calibrated to 280 nm to determine the tyrosine content present in the acid-soluble filtrate. Under specific test conditions, one unit of alkaline protease activity was defined as the quantity of enzyme that released 1 µg tyrosine ml^{-1} min^{-1} from casein.

Optimization of the medium component: One variable at a time (OVAT)

Incubation period and pH

The production medium was inoculated with 3.0 mL of fresh culture suspension of *Nesterenkonia* sp. K-15-9-6 of optical density 1.0 at 660 nm and incubate them at 37°C for 120h. To check the production of hydrolytic enzymes 10mL of the sample was collected at every 24h of intervals and supernatant was collected after centrifugation at 10000 rpm for 10 min. The enzyme activity was measured as per the method mentioned above. Similarly, the effect of pH was analyzed by arranging the medium pH 7.0, 8.0, 9.0, 10.0, and 11.0. The pH was set after autoclave using a sterile 20% Na_2CO_3 solution. The supernatant was collected after centrifugation at 10000 rpm for 10 min and used as a crude enzyme to estimate the production of hydrolytic enzymes.

Gram waste concentration

The composition of the substrates and the process variables influence the enzyme synthesis in the shake flask method. Both are initially optimized by OVAT to explore the impact of different process parameters on the synthesis of amylase, xylanase, cellulase, lipase, and protease. The range in which they affect the process and gram waste

concertation was studied in the range of 2-10g%. This approach included altering a single parameter while keeping the other elements constant. Next, the set of experiments was incubated for 48h at 37°C and from the supernatant, all the hydrolytic enzyme production was measured using the above-mentioned procedure.

Inoculum volume and medium volume

The effect of inoculum volume and medium volume on the co-production of hydrolytic enzymes was estimated in the range of 3.0-15.0% v/vand 25-150 ml/250 ml flask, respectively. The optical density (1.0 at 660nm) of *Nesterenkonia* sp. K-15-9-6 was maintained and inoculatedin different volumes in the production medium. Similarly, Medium volume (same composition of the nutrient) was varied in the production medium and incubated for 48h at 37°C.The supernatant was collected after centrifugation at 10000 rpm for 10 min and used as a crude enzyme to estimate the production of hydrolytic enzymes.

NaCl concentration

As theisolate is moderator halophilic in nature the NaCl concentration is optimized to increase their growth and enzyme production.The medium was prepared with different concentrations of NaCl (0-15.0g%). Next, the 3.0%v/v prepared K-15-9-6 suspension was used as inoculum for the experiment. After incubation at 37°C for 48h, the supernatant was collected and used as a crude enzyme to estimate the production of hydrolytic enzymes.

RESULTS AND DISCUSSION

Optimization of the medium component: One variable at a time (OVAT)

Incubation period and pH

The time course of K-15-9-6 was analyzed for up to 5 days for co-production of enzymes. The results indicate, that 48 h is the most appropriate incubation time that produced maximum hydrolytic enzymes in a single medium. However, over-incubation can reduce the synthesis of the enzymes. For example, cellulase (6.3 U/mL), amylase (5.08 U/mL) and amylase (3.76 U/mL) released maximally after 72 h of incubation, while protease (53.35 U/mL) and lipase (1.45 U/mL) produced in maximum amount after 48 h of incubation (Fig. 1). Further incubation up 120h indicating drastic change in concentration of enzyme secretion. As the lower time with a minor difference in yield favours the formation economy, hence 48h incubation period is considered for further study. The pH variation on co-production of enzymes suggested, the K-15-9-6 produced maximum hydrolytic enzymes in alkaline pH range, while acidic, neutral, and extremely alkaline (pH >11.0) reduced the cumulative biosynthesis of enzymes(Fig. 2). The most favourable pH is pH 9.0 for co-production of protease (96.54 U/mL), lipase (1.5 U/mL) and xylanase (5.3 U/mL), while amylase (5.2 U/mL) and cellulase (3.5 U/mL) produced maximally at pH 8.0. There are several bacterial, actinomycetes and fungal species have been isolated by various researchers that grow and produce different hydrolytic enzymes in alkaline pH. Concerning time, bacteria require less time for the fermentation process compared to actinomycetes and fungi (Chugh et al., 2016; David et al., 2018; Rathore and Singh, 2021).

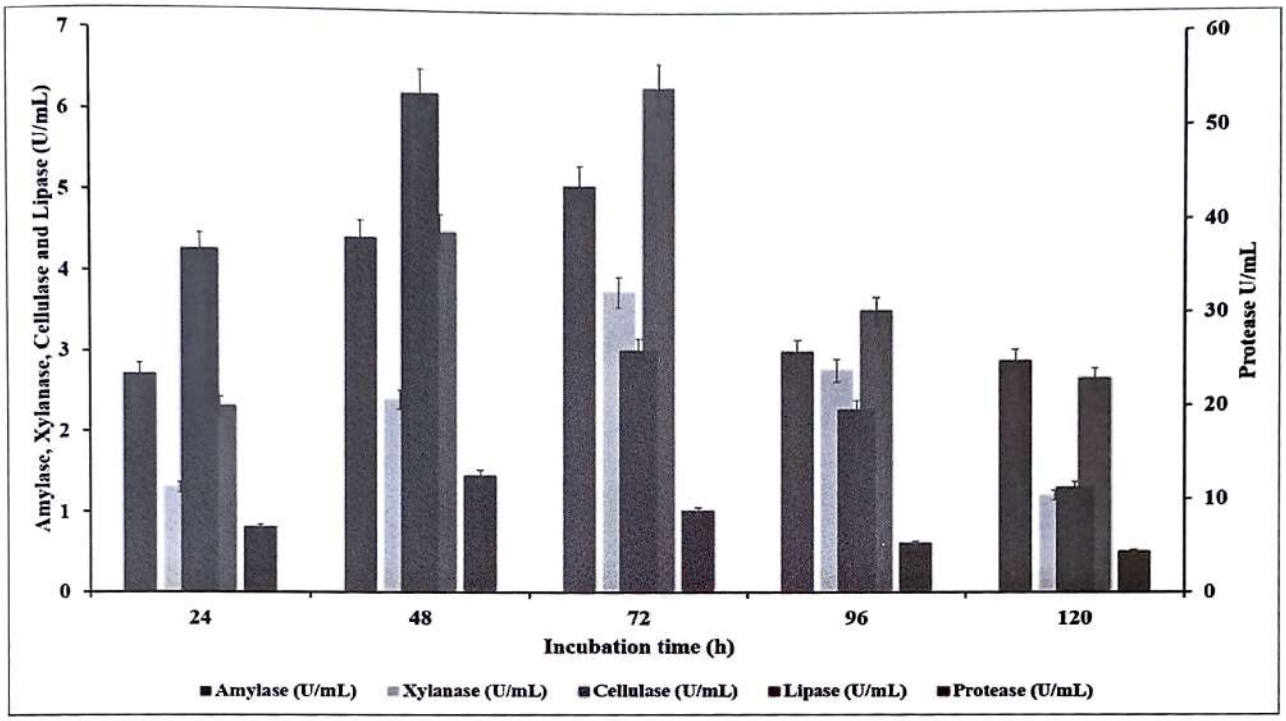

Fig. 1 Effect of incubation time on co-production of hydrolytic enzymes

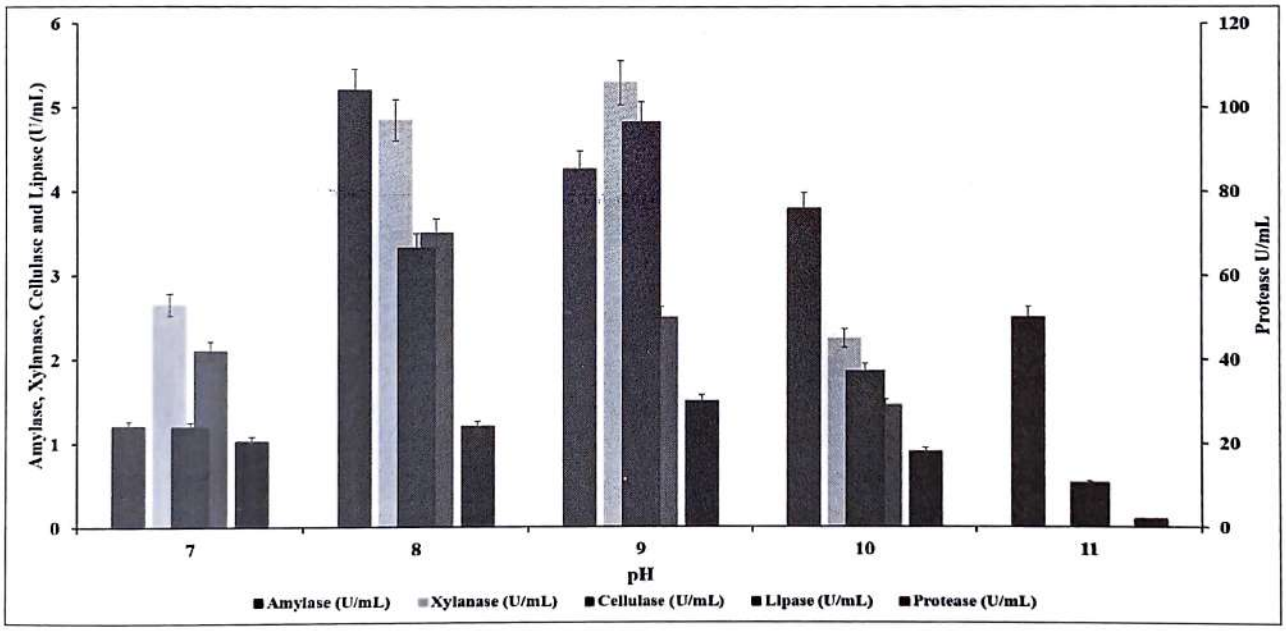

Fig. 2 Effect of pH on co-production of hydrolytic enzymes

Gram waste: Cost-effective substrate for the co-production of hydrolytic enzymes

Gram waste was tested for the co-production of hydrolytic enzymes. Experiment results indicate that gram waste supports the simultaneous production of amylase, xylanase, protease, cellulase, and lipase. As the literature indicates the gram waste contains a high amount of protein, which enhanced the protease production up to a

maximum 111.5 U/mL at the concentration of 8.0w/v%. While the other hydrolytic enzyme production was observed in lower quantity. The amylase produced 1.1U/mL, xylanase produced 3.9U/mL, cellulase produced 3.72U/mL, and lipase produced 1.45U/mL (Fig. 3).

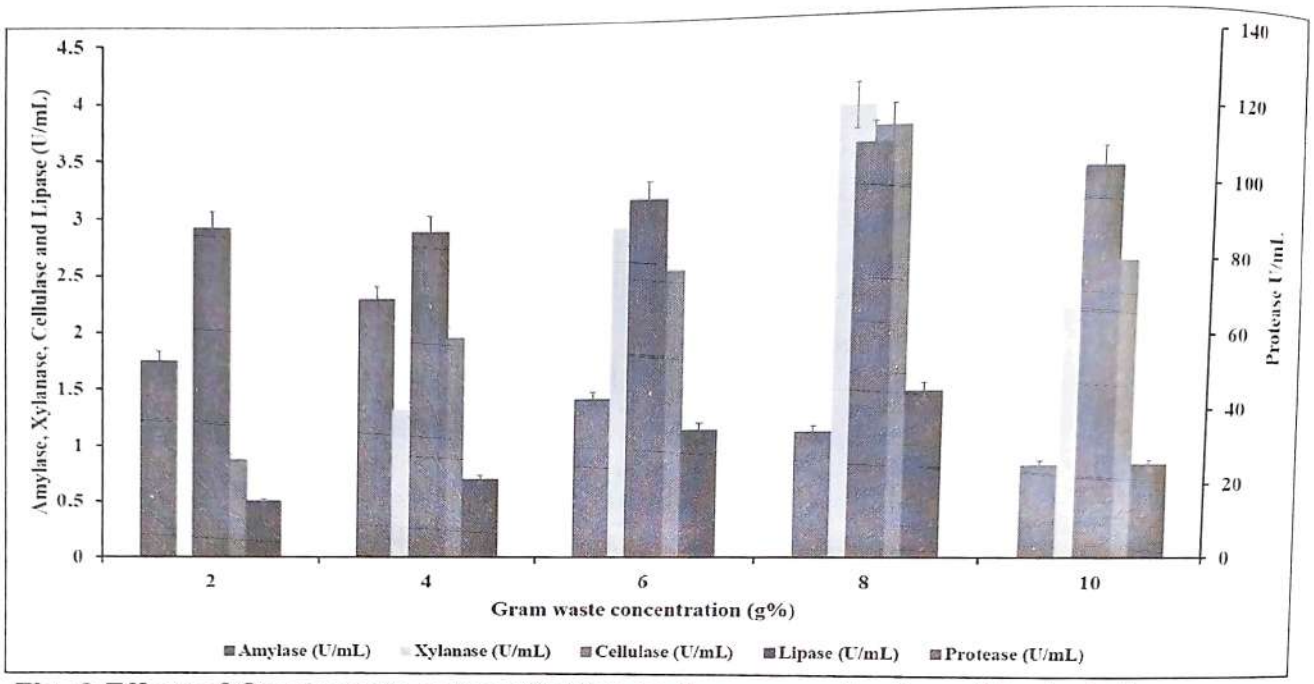

Fig. 3 Effect of Gram waste concentration on the co-production of hydrolytic enzymes

The synthesis of all enzymes is lowered by the decreasing content of gram waste. When compared to other waste sources, gram wastes are preferred as an economical and nutrient-rich raw material for the production of enzymes. Gram by-products, such as bran and husks, are a great substrate for bacterial fermentation because they have a unique composition of lipids, proteins, fibers, carbs, and bioactive substances (Prakasham et al., 2006; Rodríguez Couto, 2008). The medium's nutritional richness is attributed to the high protein and carbohydrate content, which supply energy for microbial development. Due to its widespread cultivation, waste materials are produced in substantial and reliable quantities. The circular economy and trash reduction concepts are in line with the sustainable feature of using gram waste (Rodríguez Couto, 2008; Suganthi et al., 2011). Since the enzymes are useful in industrial processes like detergent, food processing, and biofuel generation, the composition of gram wastes facilitates their biosynthesis. These enzymes include protease, amylases, xylanases, lipases, and cellulases. Numerous scientists have employed agro-food wastes to co-produce enzymes. For example, potato peels (PP) (Tuysuz et al., 2020), distillers dried grains with soluble/s (DDGS) (Chugh et al., 2016; Cekmecelioglu and Demirci, 2020), feather meal, PP and rape seed cake (Bhange et al., 2016), wheat bran (David et al., 2018), and cottonseed oil and soybean meal (Niyonzima and More, 2014) and other natural organic waste such as DORB, PP, bagasse, cereal bran, husk, fruit peels & pomace, kitchen waste and sea-food wastes for the co-production of hydrolytic enzymes via fermentation (Arya et al., 2022a).

Inoculum volume and medium volume

An inoculum volume was estimated in the range of 3.0-15.0%v/v, among them 5%v/v inoculum showsthe highest co-production enzymes. While higher inoculum (15.0%v/v) reduced the production of hydrolytic enzymes. Fig. 4 demonstrates, at 5.0%v/v inoculum maximum of 98.1U/mL of protease, cellulase 3.7U/mL,and xylanase 3.5U/mL, however, 3.0%v/v inoculum favours the amylase production (2.42U/mL). Also, Fig. 5 describes that 7.0%v/v inoculum enhanced the lipase production upto 1.7U/mL.This behaviour can lead the phenomena like higher/lower cell density in limited nutrient quantity. At the same time, the volume of production medium is optimized. The results indicate that 75mL of medium volume is most appropriate for higher production of protease (101.5U/mL), amylase (2.5U/mL), xylanase (3.69U/mL), and lipase (1.9U/mL), while, maximum cellulase (3.78U/mL) production is observed with 50mL production medium. During microbial fermentation, the inoculum volume and medium volume are crucial factors that can significantly influence the overall efficiency and productivity of the fermentation. It is essential for achieving maximum productivity, minimizing the risk of contamination, and ensuring efficient utilization of resources. These optimizations contribute to the economic feasibility and sustainability of enzyme production processes in the context of utilizing agro-food wastes (Kennedy and Krouse, 1999; Arya et al., 2021).

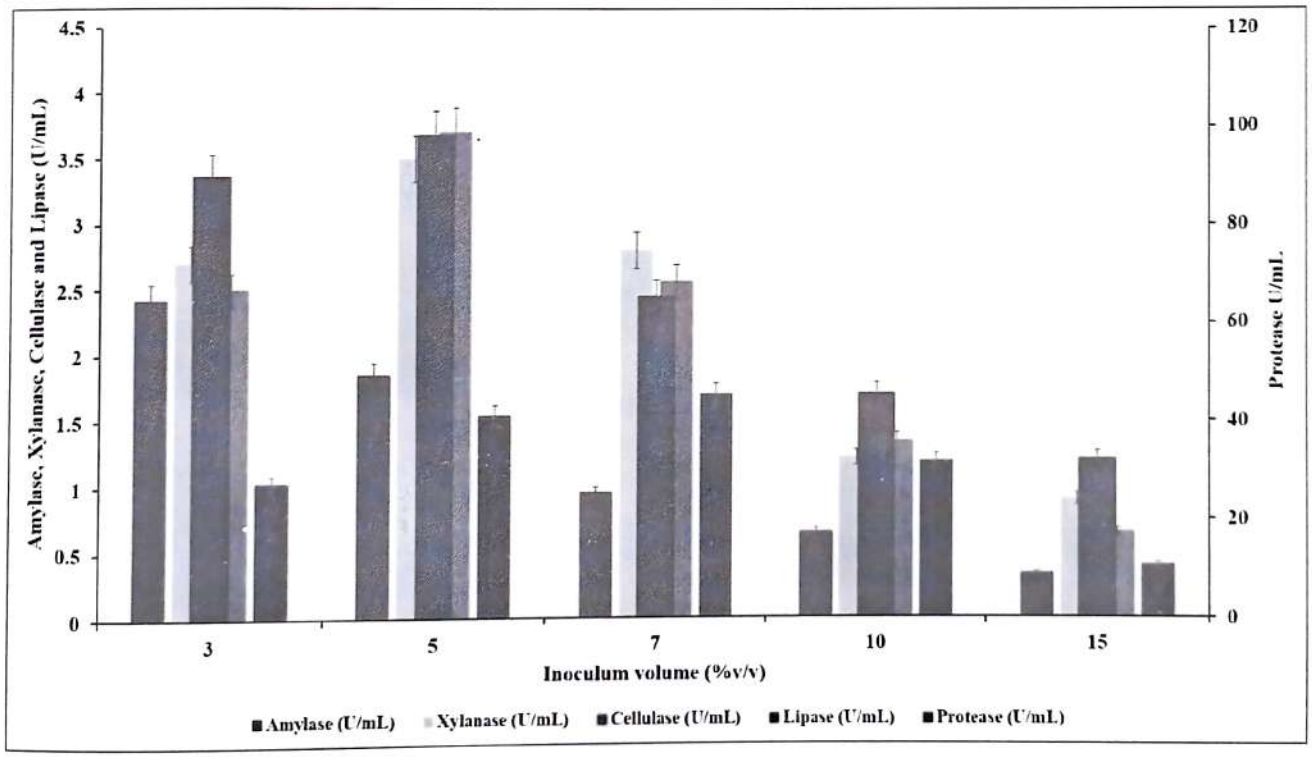

Fig. 4 Effect of inoculum volume on co-production of hydrolytic enzymes

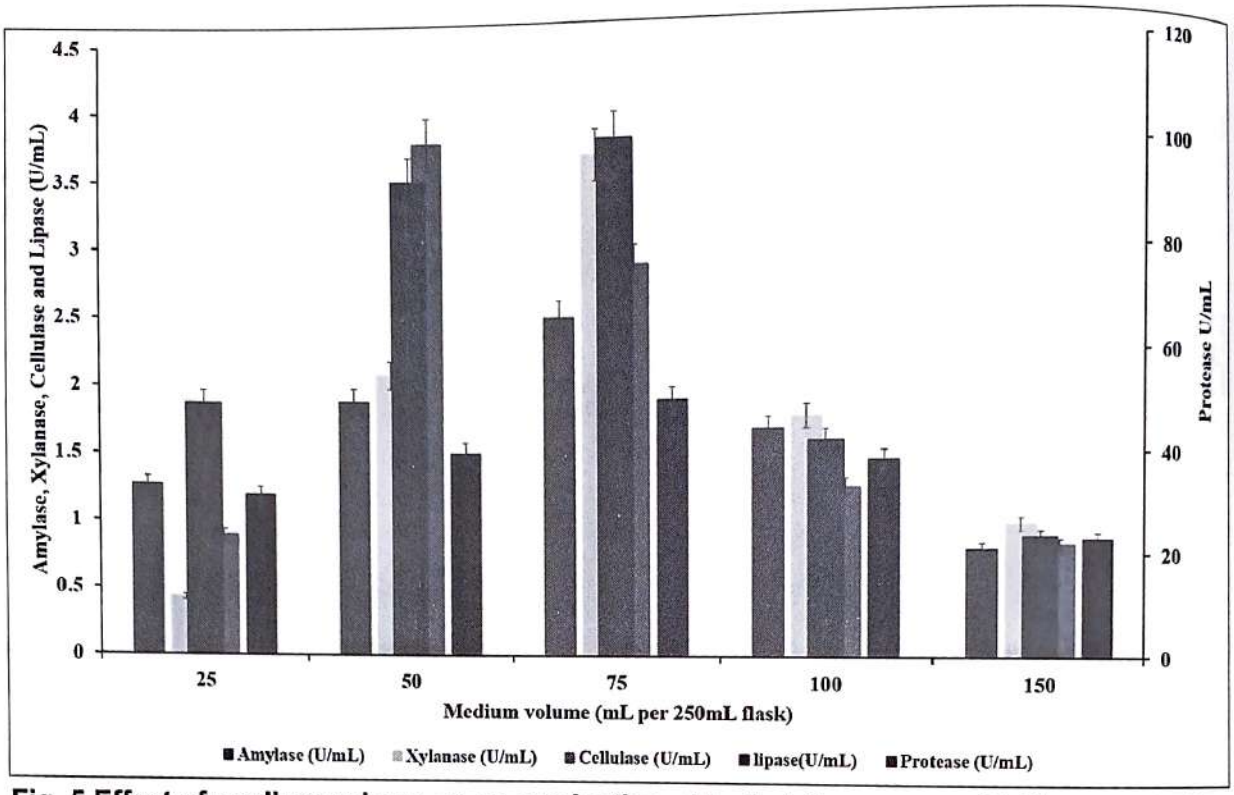

Fig. 5 Effect of medium volume on co-production of hydrolytic enzymes NaCl concentration

Furthermore, it has been documented that halophilic microorganisms may promote the synthesis of enzymes in an alkaline pH range of 8.5 to 11, in the presence of NaCl in the 0–15.0% (w/v) concentration range, as opposed to neutral and acidic conditions (Raval et al., 2014). As the isolate is mordant halophiles, at 2.5w/v% NaCl concentration all enzymes are produced in maximum amount (Fig. 6). However, the growth and enzyme production was not observed without NaCl in the medium. Similarly, the higher concentration of NaCl is also not favour for the co-production of hydrolytic enzymes. During the fermentation, protease, amylase, xylanase, cellulase, and lipase are produced maximally up to 129, 5.89, 5.12, 5.66, and 4.99U/mL, respectively. Enzyme induction or suppression by certain compounds may be influenced by environmental variables that affect the production of extracellular hydrolytic enzymes. The production of enzymes was affected by several factors, including pH, temperature, incubation period, aeration-agitation rate, and inoculum volume (Arya et al., 2021; Arya et al., 2022b). Most marine bacteria need an alkaline pH range and a mesophilic temperature range to produce enzymes; however, their production activity is reduced at very low and high pH, salt, and temperature ranges. It also depends on the source of the bacterial isolate. However, this extremophile releases a variety of enzymes that give it a unique capacity to function in such an environment (Dalmaso et al., 2015).

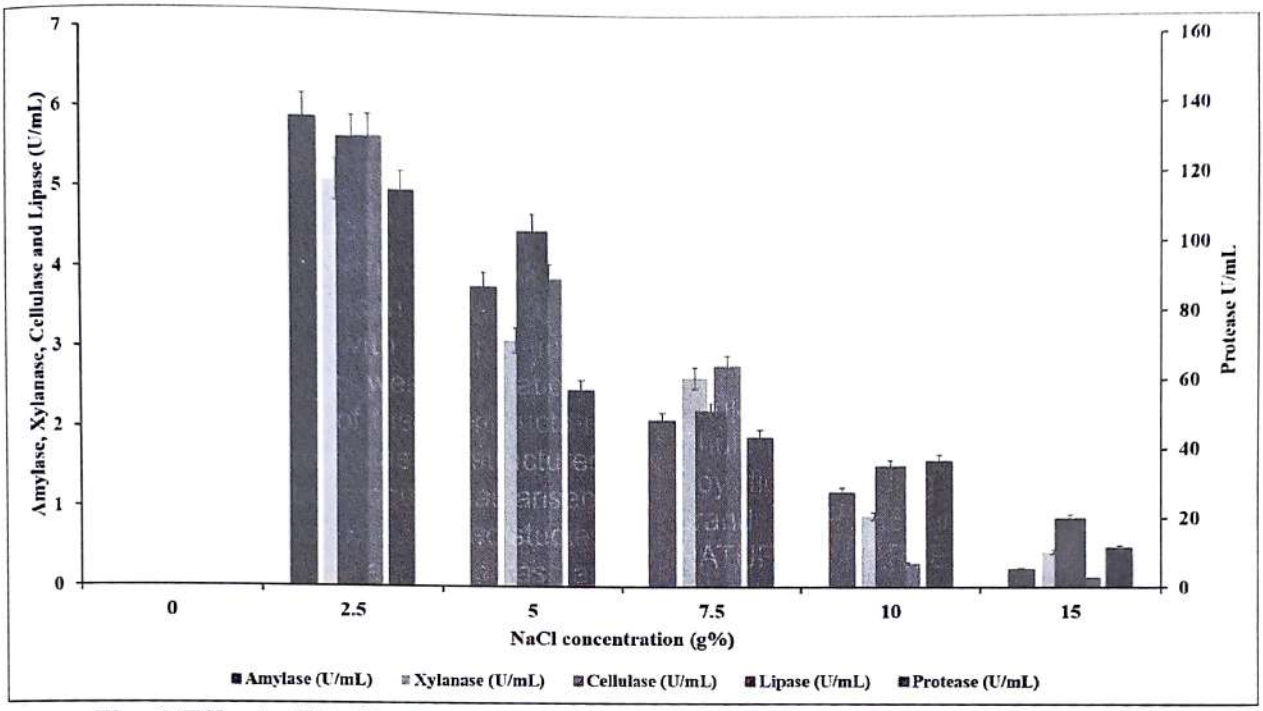

Fig. 6 Effect of NaCl concentration on co-production of hydrolytic enzymes

CONCLUSION

In conclusion, the global waste crisis spurred by population growth demands innovative approaches to sustainable waste management. Microbial enzyme synthesis offers a transformative solution. This study on *Nesterenkonia* sp. K-15-9-6 demonstrates simultaneous synthesis of various enzymes via submerged fermentation, utilizing cost-effective agro-food wastes, particularly gram waste, showcasing promising avenues for sustainable waste management. Co-production streamlines enzyme synthesis, enhancing efficiency and reducing costs. The optimal conditions-incubation time: 48h, pH: 9.0, gram waste: 8g%, inoculum volume: 5%v/v, medium volume: 75mL, NaCl concentration: 2.5%w/v-yield the maximum protease (129 U/mL), xylanase (5.12 U/mL), cellulase (5.66 U/mL), amylase (5.89 U/mL), and lipase (4.99 U/mL). This study underscores *Nesterenkonia* sp. K-15-9-6 potential for sustainable gram waste management and enzyme co production. This study not only contributes to the understanding of enzyme production dynamics but also opens up promising avenues for the valorization of agro-food wastes, paving the way for sustainable and economically viable waste management strategies in the future.

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