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ORIGINAL ARTICLE

Screening and Enhanced Production of neutral invertase from *Aspergillus* sp. by utilization of Molasses – A by-product of Sugarcane industry

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ABSTRACT
Limited studies have been dedicated to neutral invertase production from fungi especially of the extracellular type. *Aspergillus* sp. a biotechnologically important strain was isolated from sugarcane soil region, exhibited high activity. From the morphological characterization and 18S rRNA nucleotide sequencing (about 1.5 kb) it was identified as *Aspergillussojae* strain PRK-2 (GenBank entry: KJ939684.1). The filamentous fungus was grown under submerged fermentation (SmF) culture conditions and optimized for elevated enzyme production. The agro-industrial liquid product, molasses served to be not only a carbon source but also behaved as the substrate for the enzyme release. Neutral pH and thermostability of invertase served as unique attributes, suggesting the applicability in industrial processes. This paper deals with screening and isolation of a potent fungus producing extracellular invertaseat a neutral pH and optimization for economic enzyme production by using molasses.
KEYWORDS: *Aspergillussojae*sp., extracellular, molasses, neutral invertase, submerged fermentation

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INTRODUCTION

The glycoside hydrolytic enzyme which is designated with the enzyme classification number EC 3.2.1.26 is β-fructofuranosidase. It bears the systematic name as β-D-fructofuranosidefructohydrolase but commonly referred as invertase or saccharase [1,2]. Invertases are capable of catalysing β (2-1) glycosidic bond of substrate sucrose resulting in an equimolar mixture of D-glucose and D-fructose [3]. This invert mixture has a nature of hygroscopicity and lower crystallinity than sucrose which enables the food products to remain fresh, moist and soft for longer spans. Enzymatic hydrolysis contributes to colourless version of products that poses to be advantageous in food industries and confectionaries [4]. Invertases are applicable in the pharmaceutical sectors for preparation of cough syrup, probiotics, digestive-aid tablets and various uses [5]. Other associated applications of the enzyme include preparation of artificial honey, animal feed, soft-drinks, beverage and baking industries. Invertase has also seen its use in paper and pulp industries, cosmetics, textile, plasticizing agents, biosensors and detection of pathogen has been reported [6-8].

Invertases are classified as acidic (pH 4.0 – 5.5), neutral (pH 6.0 – 7.0) or alkaline(pH 7.0 and 8.0) forms of enzymesbased on the pH optima [9,10].Acidic invertases are among the popularly studied and well known invertases especially from plants and several yeast strains. On the other hand, neutral invertases (NIs) are typically characterized in plants and least in microorganisms, there is not much information about neutral/alkaline invertases at thenative protein level. However, there have been reports on cloning

and expression in *E.* coli [11]. NIs are regarded to depict superior characteristics than invertases of *S. cerevisiae* strains, indicating that it can be potentially applied in industrial divisions.

Microorganisms, plants and some animal tissues are natural producers of invertase [12]. Microbial sources of invertase reported are yeast, fungi and bacteria [13-15]. Invertase from filamentous fungi is now being globally explored. The most interesting feature of filamentous fungi is that they are easy to cultivate or grow, dynamic production of extracellular enzymes and it is of potential importance for large scale industrial purposes [16]. Submerged fermentation (SmF) is employed for invertase production for many cultures [17-19], while solid-state fermentation (SSF) is less explored. *Saccharomyces cerevisiae* [20], Aspergillusniger [21], *Fusariumsolani*[22] are some studies reporting extracellular liquid cultivation.

Invertase has been studied in extensive aspects and this is chiefly true in case of yeast invertases. Majority of research concerning yeast invertase has focused on *Saccharomyces cerevisiae*sp., which has absolutely neglected the enzyme potential of bacteria, fungi or other non-yeast microorganisms. There still arises a need to investigate microorganisms producing extracellular invertases which are useful in industries, although the *Saccharomyces cerevisiae* sp. has been abundantly worked so far [14]. Thus, there is a dearth of attention for invertase production from filamentous fungi especially extracellular types possessing exclusive characteristics,and this is of great importance in industrial sectors [19]. Kurakake et al. [23] reported that filamentous fungi have attracted remarkable consideration as notable invertase producers.

*Aspergillus*sp. has been described to be a credible producer of different enzymes possessing properties which are preferred in biotechnological and many industrial functions. There are a few reports of *Aspergillus*sp. reported for production of invertase which demands a great deal of consideration. The present study highlights screening, isolation and production a potent NI from *Aspergillussojae*strain PRK2 by SmF, which poses to have characteristics applicable to industries.

MATERIALS AND METHODS

Selection, isolation and identification of invertase producing fungus

The fungal isolates were screened for extracellular invertase production from soil sources particularly from sugarcane fields that are deemed to be sucrose rich. 1 g of the biological material was suspended in 10 ml of sterile distilled water and serially diluted up to 10^{-4} dilutions. The diluents were spread plate on sucrose agar isolation medium [24] of pH 6.5and incubated at room temperature. The fungal colonies were inoculated repetitively on potato dextrose agar (PDA) for isolated strains. Furthermore, in order to determine invertase activity by the isolates each of these isolated colonies was inoculated in sucrose broth medium, the same medium conditions devoid of agar. Fungal strains possessing high enzyme activity was chosen as potential invertase producers. The most potent isolate was morphologically identified by staining with lacto phenol cotton blue (LPCB) stain and further 18S rRNA molecular sequencing was conducted by an authentic agency, Xcelris Labs Ltd. (Ahmedabad, India).

Optimization of influencing process parameters under submerged conditions

To determine the most suitable day for maximum invertase production, the time course was studied for duration of 6-7 days. The fermentation medium constituted an initial pH of 6.5 served as control, while the effect of pH of culture medium was examined by adjusting the medium pH to 4.5, 5.5, 6.5, 7.5 and 8.5 with a digital pH meter. The influence of incubation temperature was evaluated at different temperatures ranging between 28 to 55°C. Varying concentrations of fungal spore suspension (1 to 5%, v/v) was examined to determine maximum activity. Organic nitrogen sources used were yeast extract, peptone, beef extract, casein, soyabean meal and inorganic sources included ammonium chloride, ammonium sulphate, urea and sodium nitrate were tested at 0.5% (w/v). The control consisted of all the nitrogen sources used in the primary medium. Evaluation of optimum concentration of nitrogen source was tested from 0.5 to 3.5% (w/v), using 0.5% as control. Molasses was used as the sole carbon source and tested at concentration ranging from 1 – 5% (v/v), to determine the optimum concentration. Determination of reducing sugars by Miller (1959)[25]and carbohydrate content as per Nielsen [26] was performed prior to molasses use. Medium without a surfactant served as control while tween 20, triton-X-100 and sodium dodecyl sulphate (SDS) were tested at 0.5% (v/v) for elevation of extracellular invertase activity.

Obtainment of crude enzyme extract

The culture medium possessing all the crucial optimized fermentation factors was sterilized at 120 °C, 15 psi for 20 min. The inoculated medium was incubated for 72 h. Furthermore, the extracellular enzyme was harvested by centrifugation of the culture medium at 10, 000 rpm for 10 min (4 °C). The supernatant was collected as the extracellular crude enzyme.

Quantification of invertase activity and protein content

The enzyme activity was estimated as per Miller [25] with slight modifications. In brief, the reaction mixture consisted of 0.5 ml of sucrose (1%) prepared in 100 mM sodium acetate buffer (pH 4.5) with 0.1 ml of suitably diluted enzyme and incubated at room temperature for 30 min. The reducing sugars were quantified using dinitrosalicylic acid (DNSA) method at 540 nm. One unit of invertase activity is defined as the amount of enzyme required to release 1 µmol of reducing sugars per min per ml under the assay conditions. The units of activity are represented as U/ml. The determination of protein was performed according to Lowry *et al.* [27] with BSA as a standard.

Statistics

The statistical analyzes were performed using SPSS (IBM SPSS Statistics 20). The values are represented by mean ± standard deviation. The Duncan's multiple range test was used to test for significant differences between the analyzed groups. The differences were considered significant at $p<0.05$.

RESULTS

Screening and identification for potential neutral invertase producer from soil

The soil from the sugarcane fields of Maddur in Karnataka state exhibited some interesting fungal isolates with invertase activity. About 35 isolates were screened for neutral invertase production, of which isolate IS-02 demonstrated good activity. The potent isolate was mounted by LPCB staining method and displayed colony characteristics which typically appeared as pale green to dark green with the maturation of conidia and seemed like a felt bed of conidiophores. This isolate was identified as *Aspergillus*sp. based on morphological and microscopic characteristics and further confirmed as *Aspergillussojae* PRK-2 by18S rRNA molecular sequencing performed by Xcelris Labs Ltd. (Ahmedabad, India).

Optimization studies for enhancedNI production from *Aspergillus* sp.

The optimization of the production medium for growth of the fungal microorganism was carried out by the conventional one-variable-at-a-time (OVAT) approach. All the variables were kept constant except one whose effect was investigated in every optimization study, keeping definite parameters for each experiment. The various factors influencing the production medium included the period of incubation, initial pH and temperature, inoculum level and nitrogen and carbon sources and their concentration.

Effect of physical factors

Enzyme activity was carried out to determine the highest day of maximum invertase production, which was found to be 72 h underSmF (240.2 U/ml). The rise in enzyme production was observed after 24 h. Highest levels of activity were observed typically at 72 - 96 h of incubation. Although, the enzyme activity was almost identical at these hours, a shorter time of incubation was chosen as increased time beyond 96 h led to slight fall of invertase activity (Fig. 1).

The pH value is critical in a fermentation system, numerous studies has shown that when this factor is optimum in the culture medium the enzyme activity is best expressed. In this study, the extracellular invertase activity of *Aspergillus sojae* sp. was at its peak at pH 6.5 under SmF and was found to be same as that of the control medium pH (data not shown). The pH value was similar in case of *Mucorgeo phillus* EFRL 03, as maximum activity of 39.2 (U/ml) occurred at pH 6.5 [28] and *X. dendrorhous* with an optimum pH 6.4 also exhibited high activity [29] under submerged states. Whereas, a narrow pH range of pH 4.0 and 5.5 influenced invertase productivity from *Aspergillus niger* [30].

Remarkably, the highest activity was obtained at 37 °C (327.8 U/ml) under submerged states in this study. Slight decrease in activity was observed at higher temperatures, 45 and 55 °C (Table 1). At elevated temperatures, there may be a possibility that the microorganism could release some proteases which may degrade proteins and thus affecting the yield [18]. The production medium was inoculated with different concentrations of fungal spore suspension (1 to 5%). Inoculum size of 2% (v/v) resulted in a significant elevated activity of 457.6 (U/ml)(Table 1).

Influence of nutritional sources and surfactants

Nitrogen constituent in a fermentation medium plays a vital role in influencing invertase production. Organic nitrogen extracts influenced the medium more positively than inorganic sources (Table 2). Beef extract demonstrated to be the best nitrogen source at 0.5% (w/v) with activity of 616.7 (U/ml).At this concentration ideal enzyme productivity was observed whereas, a repressive effect was detected beyond 1.5% (w/v).

Carbon sources used in the fermentation medium appears to serve even as the substrate. In the current study,molasses (2%, v/v) was used in fermentation process under submerged conditions (889.4 U/ml). Molasses as a carbon constituentwas chiefly chosen to be the sole carbon source as further addition of supplements had a negative impact on external invertase production (Fig. 2). Some studies involved the

use of molasses in a fermentation medium for invertase production. *Fusarium solani* exhibited maximum activity (9.9 U/ml) at 2% (v/v) molasses which was achieved after 96 h under SmF. 15% of molasses was optimum for *Kluyveromyces marxianus* strain [31].

Lastly, in order to facilitate the release of extracellular invertase and enhance production, tween 20 (0.5%, v/v) posed to be more suitable compared to the medium with triton-X-100 or SDS. The activity was higher (949.2 U/ml) than the control medium which lacked surfactants (Fig. 3).

DISCUSSION

The soil plays a predominant role as it is the hub for numerous microorganisms. Potent isolates can be found in natural habitats. Therefore, selection of soil samples may determine the vitality of the enzyme. Screening was performed by the plate culture method. After 3-5 days of incubation, subsequent sub-culturing for pure cultures on sucrose agar plates was performed. Furthermore, these colonies were checked for extracellular invertase production by determination of invertase activity. The colony with highest ability in hydrolyzing sucrose was chosen as the potent strain for invertase production and was further narrowed down to one single effective colony for additional investigations. Morphological characters and molecular sequencing confirmed the filamentous fungus as *Aspergillus sojae* sp. PRK-2. *Aspergillus* sp. has been described to be a good producer of various enzymes and thus the identified isolatecould be of immense industrial interest preferred biotechnologically.

The effect of optimization parameters under study was carried forward to subsequent experiments.There are several reports for determination of the ideal culture conditions for β-fructofuranosidase production from *Aspergillus caespitosus* by using the one-factor method [10, 22]. As per Lucca *et al*. [14], interaction of factors can be observed in an experimental design for optimization. But, the biological interaction of influencing parameters can be observed mostly with one factor approach.

There are several reports on extracellular invertase release under SmF conditions. *Aspergillus nidulans* and *Emericelanidulans* cultures was reported with maximum activity at 72 h of incubation [19]. In another instance, *Aspergillus nidulans* and *Aspergillus caespitosus* also demonstrated highest activity on the third day under SmF in shaking conditions [32]. *Paecylomicesvariotii*,a filamentous fungus expressed optimum production at 72 h in submerged condition [33]. While, *Mucorgeophillus* EFRL 03 showed highest enzyme activity at 48 h [28] and when *Candida utilis* was grown under SmF invertase production was achieved at 96 h [1] and also *Fusarium solani* [22]. *Aspergillus* species were good producer of invertaseswith agro-wastes as substrates such as *Aspergillus caespistosus* with wheat bran [10], *Aspergillus flavus* with pomegranate peel [34] and *Aspergillus ochraceus* with sugarcane bagasse at 72 - 96 h of cultivation [17].

pH variations can cause alteration in enzyme and/or substrate characteristics [18]. NIs are least explored, as acid invertase has been enormously studied and optimum activity is active at pH 5.0 [35]. A catalytically active neutral pH invertase is desirable in enzyme biosensors and primarily for production of invert sugar syrups [11].

Temperature is an environmental factor which affects the cellular activities, physiological events such as microbial growth, enzyme functions and nutritional requirements [36]. 30°C favoured invertase production from *Aspergillus niger* [10] and was also the ideal temperature for the growth of *Aspergillus terreus* sp. for invertase production [37]. Contrastingly, *P. variotii* demonstrated extracellular invertase release at 60 °C under SmF [33]. Lucca *et al*. [14], reported the increased levels of activity was obtained when the reaction was performed at 45°C at pH 5.0.

Hydrolytic processes may be carried out in mild conditions, thus balancing energy economy. Decline in enzymatic activities could also be due to inadequate supply of substrates or nutrients in the culture medium. When the enzyme is affected by any modifications in pH and temperature values in the environment, it may lead to alterations in the lateral chains of the amino acids thereby, interfere with the protein conformation and eventually disrupt catalytic activity [14]. The remarkable aspects of enzyme resistance to pH and temperature changes are preferable for industrial applications. Reports suggest that supplementation with nitrogen sources promoted invertase activity [10, 33]. Hence, a strong correlation lies between nitrogen equilibrium and cell productivity as described by Neto *et al*. [38]. Additionally, molasses may have also behaved as protein source, thereby influencing invertase production.

Generally, molasses possesses a variable mixture of total sugars, nitrogen and phosphorus contents [39]. Hence, it was crucial to estimate the amount of total sugars and reducing sugars before the use of molasses. Invertase production is strongly controlled by glucose repression or carbon catabolic repression (CCR) [40]. The phenomenon of CCR is predominantly seen in most cases [33, 41]. The addition of glucose to the medium often hinders the growth and release of the microorganism producing invertase.

Interestingly, there are some exceptions where CCR was not observed under fermentation conditions. Glucose did not inhibit the production of extracellular invertase by *Aspergillus ochraceus* with 35.8 (U/ml) [17]. The raise in glucose concentration up to 5 -10 g/l in a culture medium of *Aspergillus niger* GH1 [39] and 100 g/l in medium growing *Aspergillus niger* Aa-20 favoured the invertase production, but the biomass formation extremely affected external enzyme in case of *Aspergillus niger* Aa-20 [42]. Alegre *et al*. [10] explained that, when the concentration of glucose is high the secretory pathway is affected but this is not true in case of internal invertase release, which was retained in the intracellular compartments. The nature of surfactants when added to the medium beneficially elevated invertase productivity.

CONCLUSION

Optimization of medium components should be carried out autonomously under the submerged conditions. The industrial sectors desire enzymatic hydrolysis of sucrose by invertase, as the products could be a sustainable supply of carbohydrates achieved by fermentation processes. The use of agro-industrial wastes can prove to be highly economical for neutral invertase production by reducing the production-operational costs. Not only the solid-state systems, but also the submerged fermentation systems are attractive as the choice of substrates can be varied. Neutral invertase from *Aspergillussojae* PRK-2 is pH and temperature dependant. Thus, an extracellular neutral invertase from a biotechnologically important strain may be used for development of value added products in food-beverage and therapeutic sectors.

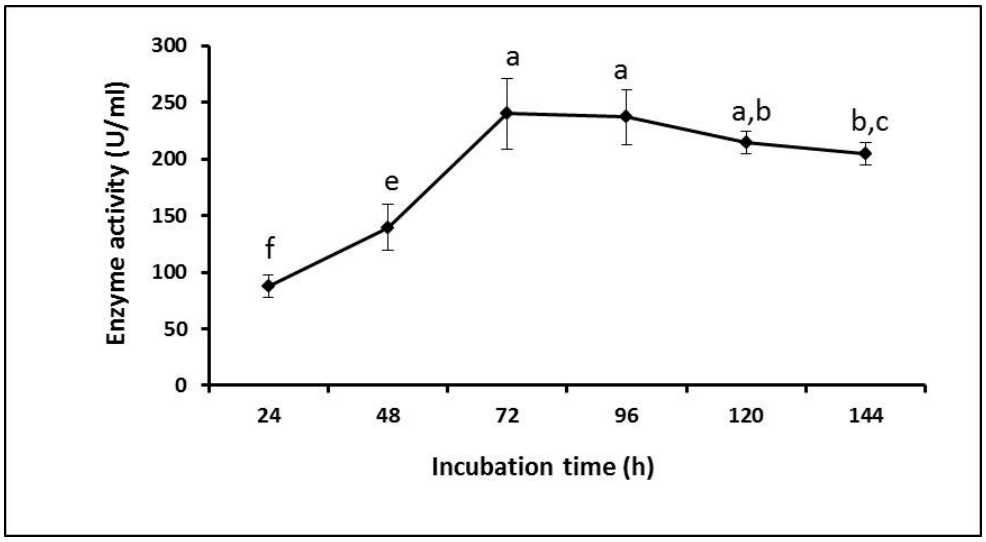

Figure 1: Extracellular invertase release from *Aspergillus* sp. at regular intervals of 24 h in submerged state.

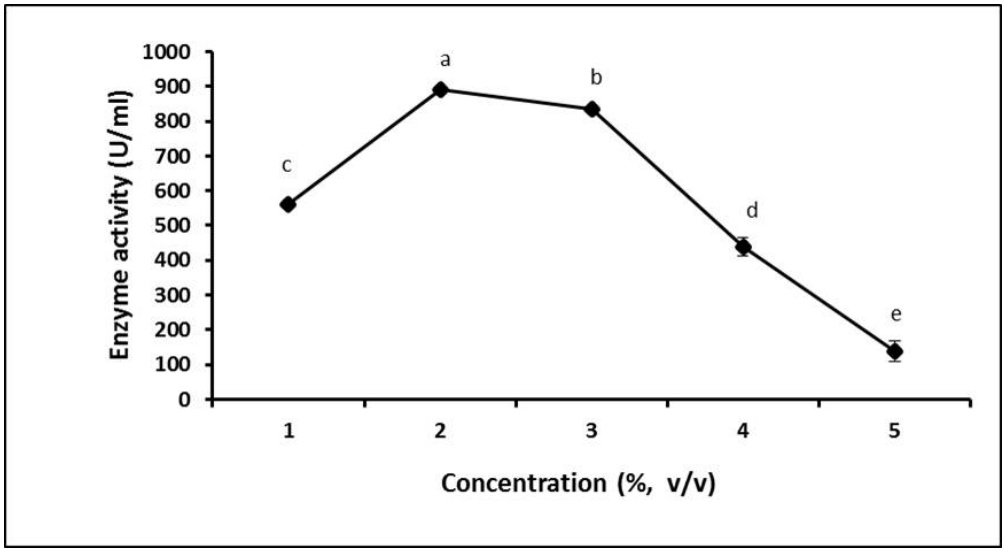

Figure 2: Concentration of molasses under submerged conditions.

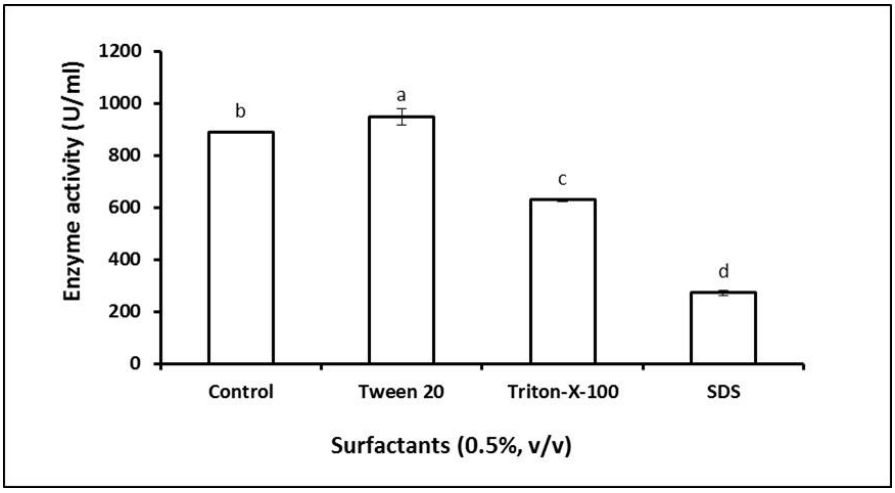

Figure 3: Effect of surfactants on invertase productivity in submerged molasses medium.

Table 1: Effect of initial temperature and inoculum level under SmF conditions

Temperature (°C)	Enzyme activity (U/ml)	Inoculum level (%,v/w)	Enzyme activity (U/ml)
28	253.19 ± 25.7^{b}	1	324.60 ± 34.2^{c}
37	327.85 ± 31.3^{a}	2	457.69 ± 9.7^{c}
45	77.91 ± 9.7^{c}	3	389.53 ± 9.7^{b}
55	29.21 ± 9.7^{d}	4	$376.54 \pm 39.3^{b,c}$
		5	$337.59 \pm 43.9^{b,c}$

Table 2: Effect of organic and inorganic nitrogen sources under SmF conditions

Nitrogen sources (0.5%, w/v)	Enzyme activity (U/ml)
Peptone	546.21 ± 9.8^{b}
Yeast extract (YE)	$396.02 \pm 11.2^{d,e}$
Beef extract (BE)	616.75 ± 14.8^{a}
Soyabean meal	$440.59 \pm 31.8^{b,c}$
Casein	233.72 ± 9.7^{f}
$(NH_4)_2SO_4$	262.93 ± 16.8^{f}
NH_4Cl	217.49 ± 5.6^{f}
$NaNO_3$	236.96 ± 31.3^{f}
Urea	344.08 ± 14.8^{e}
YE + Peptone + $(NH_4)_2SO_4$	$431.72 \pm 39.3^{c.d}$

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