Pak. J. Bot., 45(2): 681-686, 2013.

A COMPARATIVE STUDY OF NUTRITIONAL AND ENVIRONMENTAL FACTORS AFFECTING EXTRACELLULAR AND INTRACELLULAR INVERTASE PRODUCTION IN *CANDIDA UTILIS*

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Abstract

Twenty-five strains of *Candida utilis* were isolated from various varieties of V*itis vinifera* (black grapes), obtained from the local markets of Lahore. Strain KC-B10 showed maximum enzyme activity. Five media, M1, M2,M3, M4 and M5 were screened for enzyme activity with KC-B10 M1 showed maximum invertase activity i.e. 25.2 ± 0.20 U/ml extracellular invertase, 21.5 ± 0.40 U/ml intracellular invertase activity and dry cell mass 5.53 ± 0.03 g/l. The nutritional and environmental conditions were optimized for this medium using shake flask fermentation technique. After optimization of cultural conditions, the maximum enzyme activity of intracellular invertase (30.4 ± 0.47 U/ml) and extracellular invertase (49.4 ± 0.20 U/ml) with dry cell mass of 6.19 ± 0.01 g/l at 24 hrs incubation time, 1.6% (w/v) sucrose concentration, 1% yeast extract, pH 4, 30°C temperature and 200 rpm agitation rate. An increase of 49% was observed in extracellular activity while an increase of 30% was observed in intracellular activity with an increase of 10% dry cell mass. Invertase finds its uses in the confectionary and pharmaceutical industries. This study indicates that single organic source of nitrogen with a single carbon source and no other supplements are required for the optimum production of both types of invertases from *Candida utilis* KC-B10. Thereby resulting in the formation of an economically friendly optimized medium for production of invertases in Pakistan, a third world country with a fast growing population and limited resources.

Introduction

Invertase, β-fructofuranosidase (EC.3.2.1.26) belongs to the class of enzymes called glycosidases. This is responsible for the catalytic hydrolysis of sucrose to release 1: 1 monosaccharides i.e., glucose and fructose known as invert sugars (Ahmed, 2008). It catalyses the hydrolytic cleavage of terminal non-reducing β-D-fructofuranoside residues in β-D-fructofuranosides i.e., it cleaves the O-C bond of fructose instead of glucose (Haq *et al*., 2007; Kotwal & Shankar. 2009). There are two main types of invertase: intracellular and extracellular. Intracellular is produced inside the *C. utilis* cells while its extracellular counterpart is secreted in larger amounts in the outside environment (Orantes-Bermejo *et al*., 2009; Dworshcack & Wickerman 1960).

Invertase is being used by the food industry in producing chocolate covered cherries. This enzyme liquefies the sugar inside. Once the candy is manufactured, it needs at least a couple of days to a couple of weeks in storage so that the invertase can do its work. This enzyme is also used for the manufacture of artificial honey, plasticizing agents used in cosmetics, pharmaceutical and paper industries, as well as enzyme electrodes for the detection of sucrose (Kotwal & Shankar. 2009). Invertase is also being used in fermentation of cane molasses into ethanol, in calf feed preparation and also in manufacture of inverted sugars as food for honeybees (Ahmed, 2008). Invertase contributes to the anti-bacterial properties of honey. The content of invertase in honey varies according to its botanical origins; various nectars require various degrees of manipulations by bees (Orantes-Bermejo *et al*., 2009). *Candida utilis* is known as torula or *Candida* yeast. They are used in the production of nutritional supplements in animal feeds (Bekatourou *et al*., 2006). But *C. utilis* is more important as it produces the largest amounts of invertase as compared to other industrial species of yeast (Dworschach & Wickerman. 1960).

Fermentation is a process involving the mass culture of microorganism under either aerobic or anaerobic. The nutritional and environmental culture conditions have a profound effect on cell growth and productivity (Iftikhar *et al.,* 2011a). Media may be supplemented with two types of nitrogen sources: organic and inorganic (Rosma *et al*., 2006). Urea is a denaturant of invertase (Hussain *et al.,* 2010). Incubation time is a vital parameter in optimisation of conditions. It is the time during which maximum growth and product formation occurs. Inoculum size is an important factor, as it is the number of cells of the organism under study, introduced into the culture medium. It determines extent and quality of the enzyme produced. Cells have the ability of changing their metabolism according to changes in the environment (Prescott *et al*., 2000; Iftikhar *et al*., 2011b). Temperature is another important factor in growth and productivity. All microbes have a definite pH growth range and pH optimum growth range. Most fungi prefer slightly acidic pH ranges 4-6. Agitation is another major contributing factor to growth and product formation. Agitation rates as high as 900 rpms are considered suitable for biomass production while agitation rates as low as 170 rpm are more suitable for invertase production (Rosma *et al*., 2006; Almeida *et al.,* 2005; Iftikhar *et al.,* 2012a; Iftikhar *et al.,* 2012b).

The objective of the study was to optimise the nutritional and environmental conditions of media for the enhancement of extracellular invertase activity in *Candida utilis* and to compare the effect of optimization of conditions of extracellular invertase activity on its intracellular counterpart.

Materials and Methods

Microorganism: The microorganism *C. utilis* under study was obtained from KC Botany laboratory. The strain used in the study was revived on YPSA plates containing yeast extract 10 g/L, peptone 20 g/L, sucrose 20 g/L and agar 20 g/L. The colonies appeared after 2-3

days; they were sub-cultured every four weeks. Vegetative inoculum was prepared in YPS medium, for batch fermentation. It was autoclaved at 121°C at 15 lbs/ in^2 for 15 minutes. Two to three days old slants were used and 10 ml of autoclaved 0.9% saline water was added to prepare cell suspension. Two milliliters of this cell suspension were transferred to 50 ml of YPS and incubated at 30°C for 16 hrs.

Media: The media compositions (g/L) are as following
M1: Yeast extract 10, peptone 20 and sucrose 20 (Dworschock & Wickerham, 1961).

M2: Yeast extract 20, peptone 40, sucrose 20, KH_2PO_4 2 and $MgSO_4.7H_2O$ 1 (Souza *et al*., 2007).

M3: $NaNO_3$ 3, KCl 0.5, $MgSO_4.7H_2O$ 0.5, $FeSO_4.7H_2O$ 0.01, K_2HPO_4 1, Sucrose 30 (Almeida *et al*., 2005).

M4: Sucrose 40, Corn steep liquor 30, $NaNO_3$ 3, KH_2PO_4 0.5, $MgSO_4.7H_2O$ 0.05, $CaCO_3$ 2.5 (Poonawalla *et al*., 1965).

M5: $(NH4)_2SO_4$ 4, $(NH4)_2HPO_4$ 1, K_2SO_4 0.7, $MgSO_4.7H_2O$ 0.2, $CaCl_2.2H_2O$ 0.1, NaCl 0.1 (Venerova & Syhorova. 1967)

Effect of incubation time: After determining the most suitable media, incubation time was optimized. This was done by incubating the prepared Erlenmeyer flasks at 8 hr intervals, starting at 8 hrs to 72 hrs to estimate the time required for optimum growth. Then critical parameter was run at 4 hr intervals to check whether the reported incubation time was accurate.

Effect of carbon source: Invertase has the ability to act on various carbon sources, i.e., sucrose, xylose, glucose, fructose and lactose. Media was prepared using the afore mentioned carbon sources and run a control that lacked a carbon source.

Effect of inoculums size: Various volumes of inoculums were taken at 1, 2, 4, 6, 8 and 10% of inoculums were taken in triplicate flasks to determine the optimum inoculums size to obtain highest intracellular and extracellular invertase.

Effect of initial pH: The pH of a medium has significant effect on growth and productivity of a microorganism. Media, with optimized carbon source and inoculums size, were maintained at four pH levels, 3, 4, 5 and 6, using pH meter. It was maintained at initial required levels using 0.5 N HCl.

Effect of temperature: The effect of temperature on productivity and growth of *Candida utilis* at temperatures 10, 20, 30, 40 and 50°C in the optimized medium at 150 rpm for 24 hrs.

Effect of agitation rate: The effect of agitation rate on enzyme productivity and growth of *C. utilis* was studied at 50, 100, 150 200 and 250 rpm, for 24 hrs at optimized temperature in Rotary shaker *(*Daihan scientific, WIS–010, Korea).

Effect of phosphate ion: The effect of phosphate ion was studied against a control. To check effect of phosphate ion the optimized fermentation medium was supplemented with dipotassium hydrogen phosphate, dihydrogen phosphate, dipotassium hydrogen phosphate, ammonium phosphate and ammonium dihydrogen phosphate and run against a control.

Effect of nitrogen sources: Yeast extract and peptone were already being used. Firstly the effect of organic sources was observed in various combinations with sucrose. The inorganic nitrogen sources used were NH_4Cl, $NH_4H_2PO_4$, KNO_3 and $(NH_4)_2SO_4$ and their effect on growth and productivity were checked. The best one was optimized.

Extracellular and intracellular invertase: After 48 h of incubation, the fermented medium was spun at 6000×*g* for 10 min. The cell free broth was used for extracellular invertase. The harvested cells were washed with acetate buffer, collected and crushed in chilled mortar and pestle in the presence of alumina to make slurry. This slurry was spun at 6000×*g* for 10 min and the supernatant was separated from the cell debris which settled down due to spinning as pellets. The volume of the supernatant was measured and tested for intracellular invertase.

Assay method: Enzyme activity was determined by Akgol *et al*., *(*2001). One invertase unit is defined as "the amount of enzyme that releases 1.0 mg of inverted sugar in 5 mins at 35°C, pH 4.5. For invertase activity, 2.5 ml acetate buffer (50 mM, pH 5.5) and 0.1 ml sucrose (300 mM) was added into the individual test tubes. The tubes were pre-incubated at 35°C for 5 min. After the addition of 0.1 ml of appropriately diluted enzyme solution, incubation was continued for another 5 min. The reaction mixture was placed in a boiling water bath for 5 min to stop the reaction and then allowed to cool at room temperature. The blank was also run parallel replacing the enzyme solution with distilled water. To 1 ml of each reaction mixture 1 ml of DNS was added and the tubes were placed in boiling water for 5 min. Then 8 ml of distilled water was added. After cooling to an ambient temperature, volume was made up to 10 ml. Absorbance was measured at 546 nm using spectrophotometer.

Dry cell mass: This was determined by centrifugation of the fermented broth 6000 rpm for 10 minutes and decanting of the supernatant. The pellets were collected by tapping the base of the centrifuge tubes and transferring them to a dry, pre-weighted watch glass. The cells were oven dried at 80°C for 1hour. After 1 hour the watch glass was weighed again and final weight was taken to be the dry cell mass.

Results and Discussion

In the present study, the strain of *Candida utilis* KC-B10 was used for invertase production. A comparative study of both types of invertase production i.e intracellular and extracellular invertase was made on the basis of nutritional and environmental conditions. Five

media, M1, M2 and M3, M4 and M5 were evaluated for production of invertase (Fig. 1). Medium M1 contained yeast extract 1%, peptone 2% and sucrose 2% (w/v). M1 gave maximum dry cell mass (5.53 ± 0.03 g/l), intracellular invertase (21.5 ± 0.4 U/ml) and extracellular invertase (25.2 ± 0.2 U/ml) activity was shown. M3 showed minimum activity intracellular and extracellular activity at 15.5 ± 0.3 and 21.5 ± 0.3 U/ml, respectively. M1 was selected for the subsequent study for invertase production.

Enzyme activity was observed at the intervals of 8 hrs from 8-80 hrs (Fig. 2). The maximum activity was observed at an incubation period of 24 hrs (21.5 ± 0.40 U/ml intra and (27.8 ± 0.05 U/ml) extracellular activity, (4.46 ± 0.02 g/l) dry cell mass. Enzyme activity stated after an initial lag phase of 8 h until the onset of stationary phase. A decline in activity was observed during longer incubation times due to nutrient deficiency in the medium which at long incubation periods became a limiting factor this effect is also known as catabolite repression. Invertase expression in *C. utilis* is dependent upon presence of monosaccharides such as glucose and fructose, their presence in higher concentration causes catabolic repression. Incubation time period of 48 hrs was observed for *Saccharomyces cerevisiae* by Haq *et al*., (2008). Incubation times as high as 96 hrs have been reported for *Saccharomyces cerevisiae* by Rashad and Nouman (2009).

The fermentation medium was supplemented with five carbon sources such as sucrose, xylose, fructose, glucose and lactose at 2%, w/v (Fig. 3). Among the sources, used sucrose was found to be the best for invertase production in terms of both types (intracellular and extracellular) showing maximum enzyme activity (20.3 ± 0.10 U/ml) and (27.8 ± 0.01 U/ml), respectively. At this carbon source the dry cell mass (4.46 ± 0.01g/l) was observed. While xylose enhanced only intracellular invertase activity and repressed the extracellular counterpart. The effect of sucrose 0.2-2.0% (w/v) was evaluated (Fig. 4) to optimise the amount of sucrose for invertase production. Highest enzyme activity was observed at 1.6 % (w/v) sucrose, intracellular invertase (25.0 ± 0.07 U/ml) and extracellular (31.4 ± 0.24 U/ml) with dry cell mass of 5.72 ± 0.17 g/l. The dry cell mass increased even at higher percentages of sucrose, but a decrease in activity was observed at values higher than optimum. The reason behind this was that presence of higher concentration of inverted sugar caused glucose-induced repression of invertase as reported by Haq *et al.,* (2005). Dworschach & Wickerman (1960) and Cairns *et al*., (1995) reported sucrose as the best as the best inducer of invertase production in various fungi.

The quality of enzyme produced by an organism is dependent upon the number of yeast cells added to the culture medium. Flasks were inoculated with the following Inoculum sizes 1, 2, 4, 6, 8 and 10 % (Fig. 5). The maximum enzyme activity was observed at 2 % (v/v), of (26.8 ± 0.02 U/ml) intracellular, (33.3 ± 0.89 U/ml) extracellular, (5.82 ± 0.17 g/l) and the dry cell mass increased. Inoculums sizes as high as 7.8 % have been reported by Rosma & Ooi (2006) and 3 % inoculums size has been reported by Uma *et al*., (2010). There is a correlation between cell density and substrate availability, as high cell desity cultures have the ability to use alternative fermentation routes as reported by Alegre *et al*., (2009).

Invertase production is largely dependent on initial pH of the fermentation medium. The effect of pH, ranging from 3- 6 was observed (Fig. 6). Maximum enzyme activity was observed at pH 4 showing intracellular activity (22.9 ± 0.22 U/ml) and extracellular invertase (35.6 ± 0.42 U/ml). At below and above optimum pH, the less enzyme activity and dry cell mass was observed at pH value. An increase from the initial pH was observed at the end of the experiment. The reason behind this relationship is that at optimum pH, along with invertase production, there is a secretion of some anions as well as some basic proteins or in some cases uptake of cations, hence alteration in pH, consequently in enzyme activity which is pH dependent. Abrahao-Neto *et al*., (1997) and Gumaraes *et al*., (2007) reported an optimum pH of 4.5 for invertase production. Linde *et al*., (2009) reported at pH (5-6.5). Tuntapatchalern & Vananuvat (1972) reported at pH 4. Bhatti *et al*., (2010) reported the maximum invertase activity at pH 3.

Temperature is a critical factor in determination of optimal enzyme activity as yeasts are unicellular organisms and particularly sensitive to changes in environmental temperature. The fermentation medium was incubated at 0, 10, 20, 30, 40 and 50°C (Fig. 7). Maximum enzyme activity in terms of intracellular (34.6 ± 0.42 U/ml) and extracellular invertase activity (44.6 ± 0.42 U/ml) with dry cell mass (5.96 ± 0.01g/l) was observed at 30°C. Similar optimum temperature range has been reported for invertase production in *C. utilis* by Abrahao-Neto *et al*., (1997) and Quierzy *et al*., (2004). Activity was very low at temperatures below optimum.

Agitation is another critical factor in enzyme activity. The media were agitated at 0, 50, 100, 150, 200 and 250 rpm (Fig. 8). Maximum activity was reported at 200 rpm. The reason behind it is that it allows the uniform mixing of nutrients in the culture medium and collision of enzyme substrate molecules, resulting in the formation of enzyme substrate complexes. Similar optimum agitation rate has been reported by Quierzy *et al.,* (2004). Aslam *et al*., (2006) also used similar agiataion for invertase production in *Saccharomyces cerevisiae*.

The effect of both types of nitrogen sources: organic and inorganic was evaluated (Fig. 9). The organist sources were studied against a sucrose control while the experimental media were of three types: YPS, yeast extract and sucrose, peptone and sucrose. Yeast extract and sucrose gave the highest intracellular invertase activity (25.5 ± 0.05 U/ml) and extracellular (37.5 ± 0.1U/ml) with dry cell mass (6.15 ± 0.05 g/l). Yeast extract was further optimised using 0.20-1.4% (Fig. 10). The optimum intracellular activity (26.5 ± 0.05 U/ml) and extracellular activity (38.5 ± 0.1U/ml) was observed at 1 % with dry cell mass (6.15 ± 0.05 g/l). Rosma & Cheong (2006) reported the use of yeast extract as nitrogen source. Uma *et al.,* (2010) reported use of yeast extract as nitrogen source for invertase production, in contrast Aslam *et al.,* (2006) reported the combined effect of peptone and yeast extract as a suitable nitrogen source.

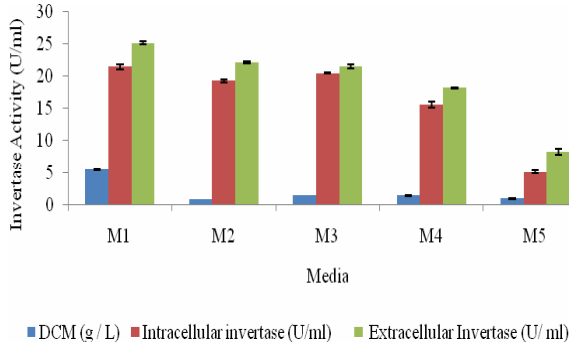

Fig. 1. The evaluation of different media for invertase production in *Candida utilis*.

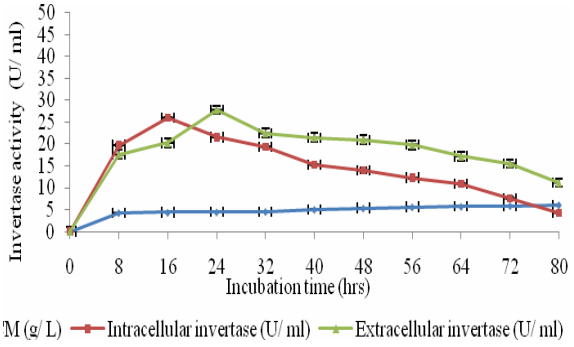

Fig. 2. The effect of incubation time on invertase production.

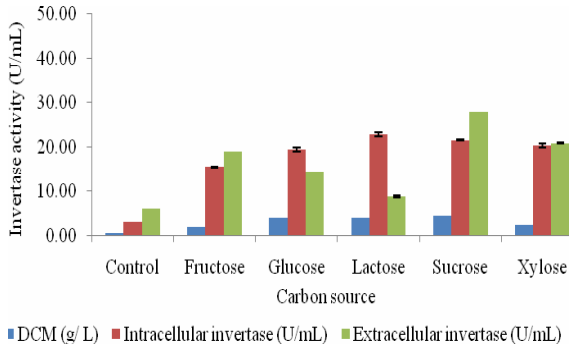

Fig. 3. The effect of carbon sources on invertase production.

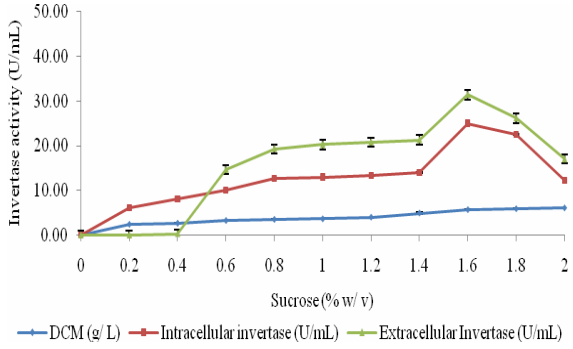

Fig. 4. The effect of different sucrose concentration on invertase production.

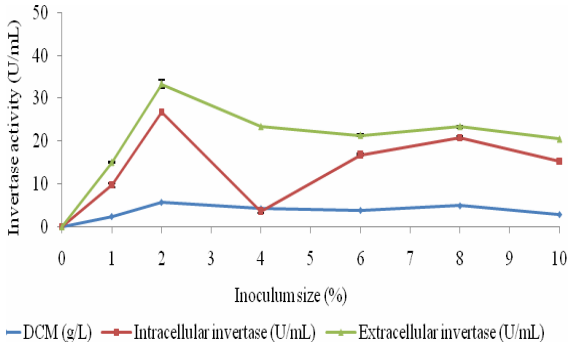

Fig. 5. The effect of different inoculum sizes on DCM, Intra and extracellular invertase.

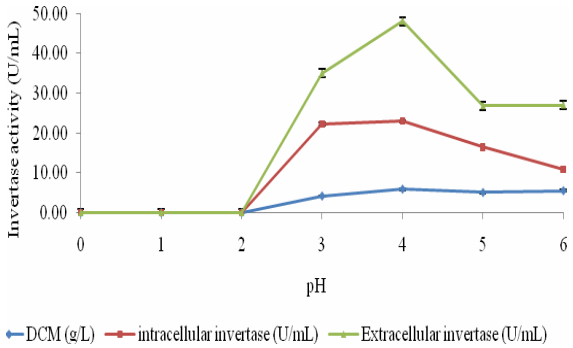

Fig. 6. The effect of pH on invertase production.

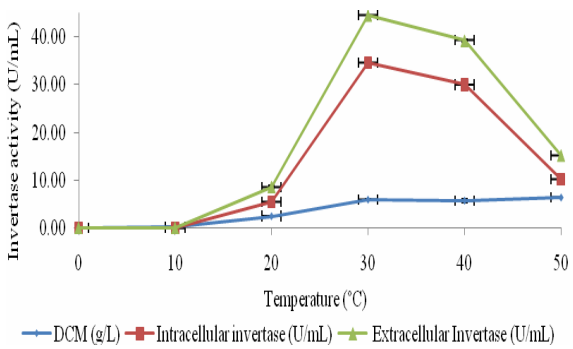

Fig. 7. The effect of different temperature on invertase production.

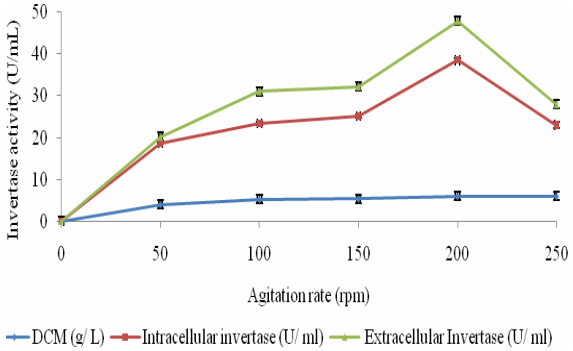

Fig. 8. The effect of different agitation on invertase production.

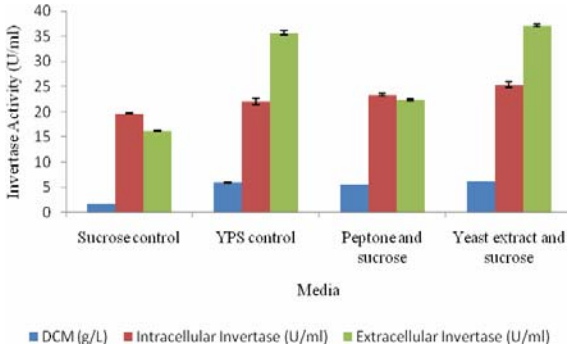

Fig. 9. The effect of different organic nitrogen on invertase production.

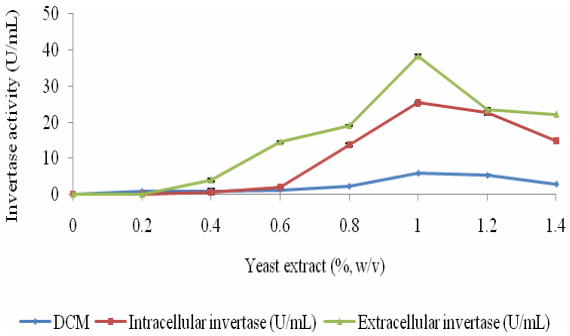

Fig. 10. The effect of different yeast extract concentrations on invertase production.

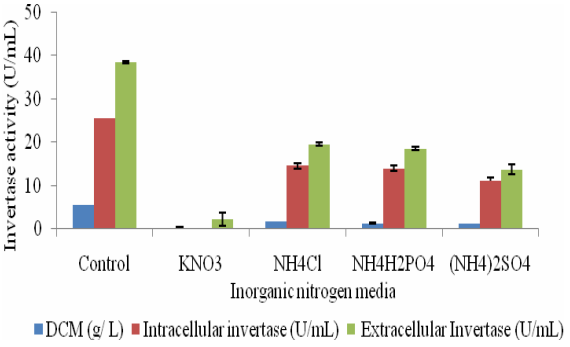

Fig. 11. The effect of different inorganic media on invertase production.

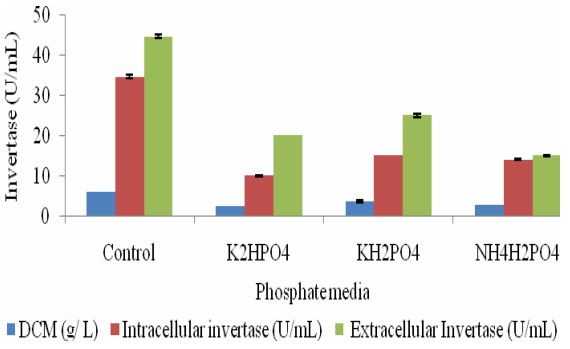

Fig. 12. The effect of phosphate on invertase production.

The inorganic sources used were: KNO3, $(NH_4)_2SO_4$, $NH_4H_2PO_4$ and NH_4Cl. These were run against a control, containing the optimised yeast extract and sucrose (Fig. 11). The control gave higher enzyme activity than the inorganic sources. The effect of phosphate sources was evaluated (Fig. 12). The phosphate sources used were potassium dihydrogen phosphate KH_2PO_4, K_2HPO_4, $(NH_4)_3PO_4$, $NH_4H_2PO_4$. These were run against the YS control. Extracellular enzyme activity of the control was higher than the phosphate experimental, while in contrast the intracellular enzyme activity was enhanced due to the presence of a phosphate source in the culture medium. In contrast, Gomez *et al*., (2000) and Shafiq *et al*., (2002) have reported enhancement of extracellular enzyme activity and biomass due to phosphate supplementation.

Conclusion

This study indicates that single organic source of nitrogen with a single carbon source and no other supplements can be used for the optimum production of both types of invertase: intracellular and extracellular from *Candida utilis* KC-B10. An increase of 49% was observed in extracellular activity while an increase of 30% was observed in intracellular activity, while an increase of 10% observed in dry cell mass after optimisation. An economically friendly optimized medium was evaluated for production of both types of invertases.

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(Received for publication 13 January 2011)